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REGULATION OF WHOLE-BODY GLUCOSE AND LIPID METABOLISM BY SKELETAL MUSCLE

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Regulation of whole-body glucose and lipid metabolism by skeletal muscle

Thesis for doctoral degree (Ph.D.)

By

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To my family

„Am Ende des Tages muss man den Ball flach halten."

Thomas Romeiser

ABSTRACT

Obesity and associated diseases like type 2 diabetes are rapidly growing health concerns across the globe. The uptake and expenditure of energy in the body are tightly regulated by a plethora of enzymes and hormones in central and peripheral tissues. Skeletal muscle is an important organ in this regulatory network and exhibits remarkable flexibility with regard to fuel utilization and modulates whole-body glucose and lipid metabolism as underlined by the work presented in this thesis.

The enzyme diacylglycerol kinase (DGK) is involved in lipid signaling and metabolism. Ablating the isoform DGKε allowed us to assess its regulatory role in whole-body energy metabolism. We observed an enrichment of diacylglycerol lipid species in skeletal muscle of high-fat fed DGKε kockout mice which was paradoxically associated with improved glucose tolerance. Nonetheless, the loss of DGKε promoted a greater whole-body reliance on lipids as fuel source. Taken together, this data identifies DGKε as a modulator of skeletal muscle lipid metabolism affecting whole-body energy handling.

Signaling of the heterotrimeric AMP-activated protein kinase (AMPK) stimulates ATP-generating processes when energy levels are low. We characterized the extent to which activity of the regulatory AMPK subunit γ1 in skeletal muscle modifies whole-body metabolism by expressing the constitutively active transgene AMPKγ1^{H151R} in skeletal muscle. This led to increased whole-body insulin sensitivity with a greater reliance on glucose as a fuel source. Furthermore, sex-specific effects on adipose tissue were observed. Our findings underline the potential therapeutic value of tissue-specific AMPK activation as it may protect against the development of insulin resistance. Conversely, the activation of AMPKγ3, another regulatory subunit isoform abundant in skeletal muscle, did not affect the whole-body lipid oxidation rate. For this assessment, we established an *in vivo* assay relying on the intravenous administration of ^{3}H-palmitic acid combined with non-β-oxidizable ^{14}C-2-bromopalmitic acid. Independently of the level of AMPK activation in skeletal muscle, we report an increased whole-body fatty acid oxidation in high-fat fed mice compared to chow fed mice.

Skeletal muscle adapts to obesity and insulin resistance by altering the abundance of certain proteins. With a state-of-the-art mass spectrometry-based workflow, we identified over 6,000 proteins in quadriceps muscle of lean and morbidly obese, insulin resistant mice lacking the satiety hormone leptin (*ob/ob* mice). Enzymes involved in lipid metabolism and proteins characteristic for slow oxidative type I muscle fibers were among the 118 differentially abundant proteins in skeletal muscle from obese in comparison to lean mice. Together with the increased abundance of proteins associated with mitochondria and peroxisomes, key organelles in the handling of energetic processes and cellular stress, this data indicates that obesity increases fatty acid oxidation in skeletal muscle.

In conclusion, the enzymes DGKε and AMPK, with its regulatory subunits γ1 and γ3, modulate skeletal muscle energy homeostasis and influence whole-body glucose and lipid metabolism. We find that obesity and insulin resistance are associated with the remodeling of the proteome of skeletal muscle suggesting increased lipid oxidation.

LIST OF SCIENTIFIC PAPERS

I. Mannerås-Holm L, **Schönke M**, Brozinick JT, Vetterli L, Bui HH, Sanders P, Nascimento EBM, Björnholm M, Chibalin AV, Zierath JR.
Diacylglycerol kinase ε deficiency preserves glucose tolerance and modulates lipid metabolism in obese mice. *The Journal of Lipid Research*, 58, 907-915, 2017

II. **Schönke M**, Myers MG Jr, Zierath JR, Björnholm M.
Skeletal muscle AMP-activated protein kinase γ1 (H151R) overexpression enhances whole body energy homeostasis and insulin sensitivity. *American Journal of Physiology - Endocrinology and Metabolism*, 309, 679-690, 2015

III. **Schönke M**, Massart J, Zierath JR.
Effects of high-fat diet and AMPK modulation on the regulation of whole-body lipid metabolism. *Manuscript submitted*

IV. **Schönke M**, Björnholm M, Chibalin AV, Zierath JR, Deshmukh AS.
Proteomics analysis of skeletal muscle from leptin-deficient *ob/ob* mice reveals adaptive remodeling of metabolic characteristics and fiber type composition. *PROTEOMICS*, In Press, 10.1002/pmic.201700375, 2018

SCIENTIFIC PAPERS NOT INCLUDED IN THIS THESIS

Kirchner H, Sinha I, Gao H, Ruby MA, **Schönke M**, Lindvall JM, Barrès R, Krook A, Näslund E, Dahlman-Wright K, Zierath JR. Altered DNA methylation of glycolytic and lipogenic genes in liver from obese and type 2 diabetic patients. *Molecular Metabolism*, 5(3), 171-83, 2016

Ruby MA, Massart J, Hunerdosse DM, **Schönke M**, Correia JC, Louie SM, Ruas JL, Näslund E, Nomura DK, Zierath JR. Human carboxylesterase 2 reverses obesity-induced diacylglycerol accumulation and glucose intolerance. *Cell Reports*, 18(3), 636-646, 2017

Younis S, **Schönke M**, Massart J, Hjortebjerg R, Sundström E, Gustafson U, Björnholm M, Krook A, Frystyk J, Zierath JR, Andersson L. The ZBED6-IGF2 axis has a major effect on growth of skeletal muscle and internal organs in placental mammals. *Proceedings of the National Academy of Sciences*, in press, 2018

Cedernaes J, **Schönke M**, Orzechowski Westholm J, Mi J, Chibalin A, Voisin S, Osler M, Vogel H, Hörnaeus K, Dickson SL, Bergström Lind S, Bergquist J, Schiöth HB, Zierath JR, Benedict C. Acute sleep loss induces molecular signatures of adverse weight gain and muscle atrophy in humans. *Manuscript submitted*

CONTENTS

LIST OF ABBREVIATIONS

ACC	acetyl-CoA carboxylase
AMPK	AMP-activated protein kinase
ANOVA	analysis of variance
AS160	Akt substrate of 160 kDa
BAT	brown adipose tissue
CPT1	carnitine palmitoyltransferase I
DAG	diacylglycerol
DGK	diacylglycerol kinase
DPM	disintegrations per minute
EDL	extensor digitorum longus
ELISA	enzyme-linked immunosorbent assay
FAO	fatty acid oxidation
FFA	free fatty acids
fl/fl	homozygously “floxed”
GIR	glucose infusion rate
GLUT	glucose transporter
GOCC	gene ontology cellular component
GS	glycogen synthase
HCD	higher-energy collisional dissociation
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	high-fat diet
HOMA-IR	homeostatic model assessment insulin resistance
ipGTT	intraperitoneal glucose tolerance test
IR	insulin receptor
IRS1	insulin receptor substrate 1
KHB	Krebs-Henseleit bicarbonate buffer
KO	knockout
LC-MS	lipid chromatography - mass spectrometry
MED-FASP	multienzyme digestion-filter aided sample preparation
MLC	myosin light chain
MYH	myosin heavy chain
NADH	nicotinamide adenine dinucleotide
ob/ob	homozygous deletion of the *obese* gene
PA	phosphatidic acid
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator-1α
PI	phosphoinositide
PKC	protein kinase C
Prkag	gene name for AMPKγ
RER	respiratory exchange ratio
ROS	reactive oxygen species

SD	standard deviation
SEM	standard error of the mean
T2D	type 2 diabetes
TA	tibialis anterior
TCA	tricarboxylic acid
TG	triglyceride
UCP1	uncoupling protein 1
WAT	white adipose tissue
WHO	World Health Organization
Wt	wildtype

1 INTRODUCTION

Over the course of the last century, our life has changed towards a more sedentary lifestyle paired with a constant availability of calorie-dense food in most parts of the world. Elevator rides to the first floor, stationary computer work and the possibility to order pizza online at 2 AM have made it unnecessary for many to use more energy than they consume throughout the day. The result is that in 2017, the WHO reported that nearly 2 billion adults in the world are overweight (body mass index (BMI) $\geq$ 25 kg/m^2) and 650 million are obese (BMI $\geq$ 30 kg/m^2) with a continuing upward trend causing this to be considered an "epidemic", a term usually only associated with quickly spreading infectious diseases. In addition to obese adults, 41 million children under the age of 5 are considered overweight or obese. Altogether this is an alarming development, given the impact of obesity-associated health impairments (WHO, 2017b). Non-communicable diseases cause 70% of deaths worldwide, ranging from 38% in low income nations to 88% in wealthy nations. Cardiovascular diseases are currently leading the ranking of the deadliest diseases (WHO, 2017d). Weight gain and obesity are caused by a positive energy imbalance over a prolonged period of time, during which the onset of metabolic diseases often goes unnoticed. Nearly 90% of all 150 million cases of diabetes in the world can be accounted to type 2 diabetes mellitus (often referred to as T2D) and this number is expected to double by 2025 due to the rise of overweight and obesity (WHO, 2017a). A better understanding of molecular processes involved in the manifestation of metabolic diseases like T2D can help combat one of the major health threats of our time and advance the development of effective treatment strategies.

1.1 TYPE 2 DIABETES AND GLUCOSE METABOLISM

T2D is characterized by a disturbed maintenance of circulating blood glucose caused by insulin resistance in peripheral tissues in combination with a relative lack of insulin. Under euglycemic hyperinsulinemia, skeletal muscle glucose uptake accounts for up to 85% of total glucose uptake from the blood (DeFronzo et al., 1981). This makes skeletal muscle one of the major organs in the regulation of whole-body glucose homeostasis. Moreover, this explains why skeletal muscle insulin resistance has detrimental effects already early in the pathogenesis of T2D (Moller et al., 1996, Zierath et al., 1998, Ferrannini, 1998).

1.1.1 Insulin action

The rise in blood glucose in conjunction with a meal stimulates the pancreatic β-cells to produce and secrete the hormone insulin into the systemic circulation. Insulin binds to the insulin receptor (IR) on the plasma membrane of the responsive cells (House and Weidemann, 1970, Ashcroft et al., 1972, Le Marchand-Brustel et al., 1978). The insulin receptor functions as a tyrosine kinase and autophosphorylates IR tyrosine residues upon ligand binding which then allows the interaction with the insulin receptor substrate 1 (IRS1) and its phosphorylation (Kasuga et al., 1982, Sun et al., 1991). This binding triggers the activation of the phosphoinositide (PI) 3-kinase pathway. The stimulation of the phosphoinositide-dependent

kinase 1 (PDK1) activates Akt which, as one of its actions, phosphorylates and hence inactivates its substrate AS160 (Akt substrate of 160 kDa, also TBC1D4) promoting the translocation of glucose transporter 4 (GLUT4) vesicles to the plasma membrane (Alessi et al., 1997, Shepherd et al., 1997, Larance et al., 2005) (Fig. 1). Glucose transport is mediated by GLUT4 and glucose is phosphorylated by hexokinase (or glucokinase in the liver) resulting in the entrapment of the charged molecule glucose-6-phosphate in the cytoplasm. Glucose-6-phosphate is now either entering glycolysis where it is converted into pyruvate and energy is released in the form of ATP or it is converted and stored as glycogen. The latter is also induced by the activation of Akt which furthermore stimulates protein synthesis via the mammalian target of rapamycin (mTOR) pathway, as well as cell survival by inhibiting autophagy (Withers et al., 1997, Sekulic et al., 2000).

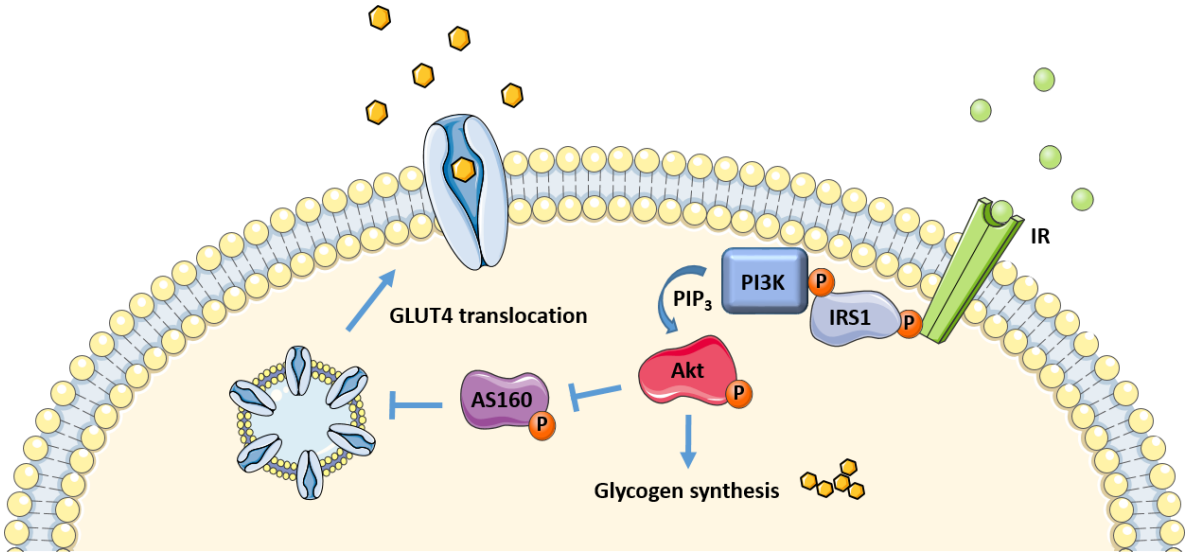

Figure 1: Insulin-stimulated glucose uptake. The binding of insulin to the insulin receptor (IR) triggers a signaling cascade through IRS1 and PI 3-kinase and results in the translocation of GLUT4 molecules to the plasma membrane allowing glucose to enter the cell.

1.1.2 Glycogen and gluconeogenesis

Since the cell has no ability to store ATP for later use, energy has to be stored in other forms. Following several enzymatic steps that metabolize glucose-6-phosphate into UDP-glucose, glycogen synthase (GS), together with the glycogen branching enzyme, build up glycogen, a cellular glucose storage molecule. The main organs storing glycogen are skeletal muscle and liver. Of these two organs, only the liver is able to break down glycogen to release glucose back into the blood during periods of fasting to maintain stable blood glucose levels. In addition, hepatic gluconeogenesis, the process of generating glucose from other sources than carbohydrates, is stimulated by the pancreatic peptide hormone glucagon and inhibited by insulin through the activation of Akt and the inhibition of the expression of the key gluconeogenic genes phoshphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (Liao et al., 1998, Kotani et al., 1999). Skeletal muscle breaks down glycogen when the energy demand within the muscle is high, for example during exercise.

1.2 SKELETAL MUSCLE, EXERCISE AND TYPE 2 DIABETES

Exercise increases the uptake of glucose into skeletal muscle cells independently of insulin. This mechanism of action is preserved in insulin resistant skeletal muscle from severely diabetic rodents (Wallberg-Henriksson and Holloszy, 1984) and in turn even enhances insulin sensitivity (Wallberg-Henriksson et al., 1988). In humans, exercise training (7 days 1 hour/day of 75% maximal oxygen consumption) increases insulin-stimulated glucose uptake and PI 3-kinase activity in skeletal muscle in comparison with the sedentary condition before training (Houmard et al., 1999). Skeletal muscle from obese type 2 diabetic subjects following a similar training protocol shows elevated GLUT4 abundance after training (O'Gorman et al., 2006). The gene expression profile of skeletal muscle is altered from hours up to several days post-exercise or muscle contraction mediating long-term effects on whole body physiology (Mahoney et al., 2005, Neubauer et al., 2014). Additionally, exercise reduces the pancreatic secretion of insulin which is a desirable effect in stages of early insulin resistance where the pancreas increases the secretion of insulin to counteract the reduced effect on peripheral tissues (Jones et al., 1997, Rynders et al., 2014). This underlines the importance of regular exercise in the prevention of T2D in people with impaired glucose tolerance and insulin resistance and treatment of people with overt T2D. The WHO currently recommends 150 minutes of moderate intensity or 75 min of high intensity physical activity per week for adults (WHO, 2017c). This recommendation takes into account that short but intense training forms like HIIT (high-intensity interval training) were shown to have similar effects on skeletal muscle physiology and overall health as longer but moderate workouts (Perry et al., 2008, Shaban et al., 2014).

1.2.1 Skeletal muscle fiber types

Skeletal muscle is composed of multinucleated muscle fibers consisting of several myofibrils. Myofibrils are subdivided into sarcomers which are repeated units of actin and myosin filaments that form the functional machinery required for muscle contraction. Upon stimulation of the neuro-muscular junction via the central nervous system, the muscle fiber depolarizes through the opening of sodium channels and calcium is released by the sarcoplasmic reticulum. Calcium binds to the protein troponin which undergoes conformational changes exposing the myosin binding site on the actin filament (Ebashi et al., 1967). The myosin filaments can now pull the actin filament closer in an ATP-hydrolyzing step resulting in a shortened muscle (Huxley and Niedergerke, 1954).

Skeletal muscle fibers are divided into several fiber types which are primarily characterized by the presence of different myosin heavy chain isoforms that define the contractile properties, fuel use and fatigability of a fiber. The main isoform in slow-twitch type I fibers with a high oxidative capacity and low but steady power output is the myosin heavy chain β that is encoded by MYH7. The moderately fast type IIa fibers that have high oxidative and glycolytic capacities express the isoform MYH2, while MYH1 expression is characteristic for fast type IIx fibers (also referred to as IId) that are used for short-term anaerobic activity. Type IIb fibers are the fastest fibers used for very short and explosive bouts of activity. They are exclusively glycolytic and characterized by the expression of the myosin heavy chain isoform MYH4 (Larsson et al.,

1991, DeNardi et al., 1993, Schiaffino and Reggiani, 2011). While the extent to which muscle fibers can interconvert remains a matter of debate, endurance training in humans leads to changes in the relative distribution of oxidative type I fibers, while strength training has an effect on the relative type II fiber distribution (Wilson et al., 2012).

Some variation in muscle fiber types exists between mammalian species. Small rodents generally have a higher proportion of fast-twitch fibers in each muscle compared to humans (Schiaffino and Reggiani, 2011). In rat skeletal muscle, several hybrid fibers were described, suggesting a continuity with these hybrids as intermediates between the different pure muscle fibers (Rivero et al., 1998). In C57BL/6J wildtype mice, the hindlimb muscle soleus was shown to contain a high proportion of oxidative slow-twitch fibers, while the extensor digitorum longus (EDL) muscle consists of glycolytic fibers. Tibialis anterior (TA), quadriceps and gastrocnemius are mixed muscles with mainly glycolytic, but also oxidative fibers (Bloemberg and Quadrilatero, 2012, Jacobs et al., 2013). The oxidative capacity is reduced in vastus lateralis muscle of T2D patients, with reduced slow oxidative and increased glycolytic fibers compared with healthy controls (Oberbach et al., 2006). However, the cause and consequence, as well as the underlying molecular mechanisms of these changes is unclear, especially regarding the abundance and functionality of skeletal muscle mitochondria in obesity or insulin resistant states.

1.3 LIPID METABOLISM

The complete oxidation of lipids, compared to carbohydrates and protein, the other two macronutrient classes, yields the most ATP per gram. Fat is mainly stored in the form of triglycerides, with three chains of saturated or unsaturated fatty acids bound to one molecule of glycerol as a backbone. In order to utilize this stored fat, triglycerides have to undergo lipolysis where the glycerol bond is digested by lipases and free fatty acids are released. This is, for example, stimulated by glucagon triggering adipocytes to secrete free fatty acids into the blood when glucose levels are low and energy is needed. Fatty acids are transported into mitochondria as acyl-CoA by carnitine palmitoyltransferase 1 (CPT1) where they undergo ß-oxidation (McGarry et al., 1978). β-oxidation produces an acyl-CoA molecule that is two carbon atoms shorter than before, an acetyl-CoA molecule that can now enter the tricarboxylic acid (TCA) cycle, and NADH as well as $FADH_2$. If acetyl-CoA does not enter the TCA cycle, it can be carboxylated by the acetyl-CoA carboxylase (ACC) into malonyl-CoA. Malonyl-CoA, in turn, is the substrate for fatty acid synthesis and inhibits the further transport of fatty acids into the mitochondria (Ruderman and Dean, 1998, Rasmussen et al., 2002).

Although lipid metabolism and the plasma concentrations of fatty acids are not as tightly regulated as the metabolism of glucose, there is a sensitive regulatory network in place involving many enzymes and inter-organ crosstalk. With metabolic dysregulation and obesity, lipids accumulate within skeletal muscle and liver which can be directly linked to insulin resistance (Kelley and Goodpaster, 2001). Diacylglycerols (DAGs) and other fatty acid metabolites activate kinases like protein kinase C (PKC), the inhibitor of nuclear factor κB kinase-B (IKKB) and the c-Jun N-terminal kinase (JNK) that phosphorylate IRS and reduce

insulin signal transduction (Samuel et al., 2004). Paradoxically, elevated levels of triglycerides can also be detected in skeletal muscle of endurance trained athletes with a high oxidative capacity (Goodpaster et al., 2001).

1.3.1 Adipose tissue

Adipocytes form one of the most dynamic tissues in the body, with the ability to expand 15-fold in size (Berry et al., 2013). With the main purpose of storing fat in times of overabundance of nutrients, adipose tissue also plays an important role in thermoregulation and the coordination of systemic metabolism. Over the last three decades, adipose tissue has been recognized as the largest endocrine organ taking part in the regulation of food intake, glucose homeostasis and fertility through the secretion of hormones, lipids and adipokines (Zhang et al., 1994, Hotamisligil et al., 1995, Hu et al., 1996, Mathew et al., 2017). Histologically, there are two main classes of adipose tissue in the body: White adipose tissue (WAT) which can be subdivided into visceral and subcutaneous depots and brown adipose tissue (BAT). In contrast to WAT, BAT is very rich in mitochondria and dissipates energy and produces heat through the uncoupling of the electron transport chain. The key characteristic of brown adipocytes is the expression of the uncoupling protein 1 (UCP1) (Nicholls et al., 1978, Lin and Klingenberg, 1980). BAT plays an important role in thermoregulation during the neonatal period, but expansion and activity of the main depot between the scapulae can also be induced in most adults through cold exposure (Cypess et al., 2009, van Marken Lichtenbelt et al., 2009, Yoneshiro et al., 2011). Due to its fat burning capacity, the induction of BAT and the "browning" of so called "beige" or "brite" adipocytes that constitute an intermediate adipocyte type within certain white fat depots have been investigated thoroughly during the last years providing a base for possible therapeutic approaches (Petrovic et al., 2010, Ohno et al., 2012, Kalinovich et al., 2017).

The location of the different white adipose tissue depots defines their response to stimuli, as well as their impact on the regulation of whole-body energy metabolism through distinct patterns of gene expression (Alves et al., 2017). The accumulation of subcutaneous fat (referring to the "pear-shaped" female fat distribution) is believed to be healthier than the accumulation of visceral intra-abdominal fat (as in the male "apple-shaped" fat distribution). Regarding the estimation of metabolic health, an evaluation of the waist-to-hip ratio rather than the BMI is (with a waist-to-hip ratio > 0.85 for women and > 0.9 for men being considered a greater metabolic risk according to the WHO (WHO, 2008)) is recommended. Thus, alterations in not only the amount, but also the distribution of fat impact metabolic health. Moreover, in mice, transplantation of subcutaneous adipose tissue into locations of visceral depots improves metabolic parameters (Tran et al., 2008). Recently, WAT has been recognized as a direct exercise-responsive tissue and the secretion of transforming growth factor (TGF) β2 from subcutaneous adipose tissue of endurance trained mice has been identified as a factor promoting exercise-related health benefits. The transplantation of subcutaneous WAT from trained into untrained mice improved metabolic parameters in the sedentary recipients leading to the discovery of this adipokine (Stanford et al., 2015). Overall, the role of adipose tissue as

an endocrine organ is not fully understood, and further studies in the context of metabolic homeostasis and exercise-responsiveness are warranted.

1.3.2 Leptin action

The satiety hormone leptin is secreted by the white adipose tissue during and after a meal, as well as during sleep. Leptin is involved in the central regulation of food intake and neuroendocrine function. Within the arcuate nucleus, located in the ventromedial hypothalamus just above the optic chiasm, leptin-sensitive agouti-related peptide (AgRP) and pro-opiomelanocortin (POMC) neurons integrate this signal and inhibit further food intake (Cheung et al., 1997, Ebihara et al., 1999, Coppari and Bjorbaek, 2012). Leptin was first discovered in the mid-1990s after the successful positional cloning of the *obese* gene that causes extreme heritable obesity in the *ob/ob* mouse strain already described in 1950 by the Jackson Laboratory (Zhang et al., 1994, Ingalls et al., 1950). When the structure of the hormone and the effect of leptin was revealed, high hopes were put into the "wonder drug against obesity". However, obesity is in most cases accompanied by leptin resistance, rather than leptin deficiency, and therefore the additional administration of leptin has little or no slimming effect (Frederich et al., 1995, Ronnemaa et al., 1997, Westerterp-Plantenga et al., 2001, Steinberg et al., 2002). Rare cases of extremely obese humans with a mutation causing the complete absence of leptin expression in adipose tissue benefit from leptin treatment to reduce weight and other neuroendocrine pathologies (Farooqi et al., 1999). In addition to the direct effect on feeding behavior, leptin exerts multiple effects on glucose homeostasis via central and peripheral mechanisms. The infusion of leptin into the cerebral ventricles reduces hepatic gluconeogenesis and increases peripheral glucose uptake (Kamohara et al., 1997, Liu et al., 1998). *In vitro* studies provide evidence for a direct effect of leptin treatment on glucose metabolism in isolated skeletal muscle cells (Harris, 1998, Bates et al., 2002). The *ob/ob* mouse line continues to be a widely used model for extreme hyperphagic obesity, insulin resistance and transient hyperglycemia on a C57BL/6J genetic background (Lindstrom, 2007).

1.4 MITOCHONDRIA AND PEROXISOMES

Mitochondria are essential for cellular metabolic homeostasis as they harbor the enzymes constituting the TCA cycle needed for the generation of ATP from glucose, lipids or proteins. Different tissues have different amounts of mitochondria with oxidative skeletal muscle, such as soleus muscle, cardiac muscle or BAT being rich in mitochondria. Besides the production of ATP, mitochondria are involved in the generation of reactive oxygen species (ROS), calcium signaling and the regulation of cell death. Mitochondrial biogenesis is stimulated through environmental stress such as exercise and the activation of the peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α) that contributes to the regulation of the expression of specific genes. Mitochondrial DNA is a circular molecule similar to bacterial DNA and contains 37 genes that encode 13 subunits of the electron transport chain complexes. However, the large majority of mitochondrial proteins are encoded in the nuclear DNA (Jornayvaz and Shulman, 2010). The most prominent member of the sirtuin family, SIRT1, has

also been linked to mitochondrial biogenesis via the deacetylation and hence stimulation of PGC-1α. This was proposed to be the mechanism behind the impact SIRT1 has on the metabolic adaptations observed during caloric restriction and longevity (Gerhart-Hines et al., 2007, Tang, 2016).

The electron transport chain located in the inner mitochondrial membrane consists of five complexes that together constitute the oxidative phosphorylation (OxPhos) system. The oxidation of NADH and $FADH_2$ from the TCA cycle to NAD^+ and FAD generates free electrons that flow through the complexes and build up a proton gradient across the membrane. The complete chain of redox reactions results in the production of water and the proton gradient drives the ATP synthase resulting in the formation of ATP from ADP and phosphate.

1.5 AMP-ACTIVATED PROTEIN KINASE

The AMP-activated protein kinase (AMPK) can be considered the main energy sensor in the cell. The enzyme is activated through elevated levels of AMP (or ADP) arising from the utilization of ATP in periods of high energy demand (Ross et al., 2016). AMP binds to the γ-subunit of the heterotrimeric complex and promotes an allosteric modulation involving an activating phosphorylation (Thr172) of the α-subunit and exposure of its catalytic site (Fig. 2, (Steinberg and Kemp, 2009)). Upon activation, AMPK phosphorylates a plethora of target enzymes inducing glucose uptake, glycolysis and lipid oxidation to generate ATP while inhibiting energy storing processes such as glycogenesis and triglyceride formation (Fig. 3). The three different subunits comprising AMPK exist in different isoforms and are encoded by different genes: The catalytic subunit (α1 and α2), the scaffolding subunit (β1 and β2) that also plays a glycogen-sensing role and the regulatory AMP-sensing subunit (γ1, γ2 and γ3) (Stapleton et al., 1996, Cheung et al., 2000, Polekhina et al., 2003). The enzyme is only functional as a triad and can exist in 12 different heterotrimeric combinations that are expressed in a tissue-specific manner and possibly exhibit different levels of activity depending on the stimulus (Mahlapuu et al., 2004, Willows et al., 2017). Together this allows a fine tuned regulation of metabolic homeostasis in peripheral and central tissues via AMPK.

1.5.1 AMPK in glucose and lipid metabolism

The acute activation of AMPK, for example during exercise, when skeletal muscle has an increased energy demand, promotes the translocation of the glucose transporter GLUT4 to the plasma membrane, allowing an increased influx of glucose into the cell (Kurth-Kraczek et al., 1999). Since AMPK simultaneously phosphorylates serine residues on the enzyme GS, the channeling of glucose into the production of glycogen is inhibited and ATP-producing glycolysis is favored instead (Wojtaszewski et al., 2002). However, the chronic activation of AMPK, for instance caused by activating mutations within the subunit genes, results in the accumulation of glycogen in the cell, as the permanently high levels of glucose-6-phosphate allosterically activate the GS presumably overwriting the direct inhibition through AMPK (Nielsen et al., 2002, Barnes et al., 2005).

While glycogen stores in skeletal muscle are needed for bursts of intense exercise, skeletal muscle mainly relies on fatty acids as fuel source during prolonged endurance exercise or when at rest (van Hall et al., 2002). AMPK is involved in the induction of lipid oxidation via the inhibition of ACC. ACCα/β promotes lipid synthesis rather than oxidation, and its inhibition by the AMPK-induced phosphorylation of the critical residue Ser79 decreases the levels of malonyl-CoA, which in turn results in reduced inhibition of CPT1, the transporter of long chain fatty acids in the mitochondria. The import of lipids into the mitochondria is now increased, elevating the β-oxidation rate (Kudo et al., 1995). AMPK furthermore exerts long-term transcriptional control of metabolic key enzymes (Long et al., 2005).

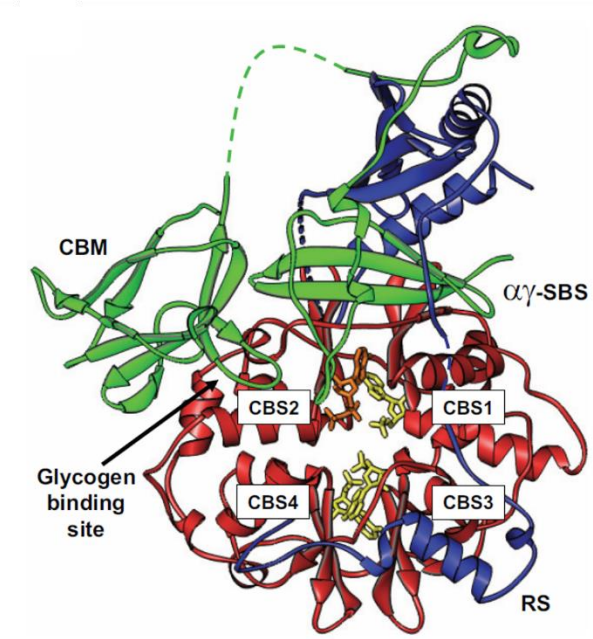

Figure 2: Graphical representation of the mammalian heterotrimeric AMPK complex and the yeast orthologs: In blue, the α-subunit with a mammalian β-subunit interacting domain (SID) and regulatory sequence possibly unique to *S. cerevisiae* (RS). In green, the β-subunit structure with a carbohydrate-binding molecule (CBM) (from *S. cerevisiae*) and αγ-subunit binding sequence (SBS) (from *S. pombe*). In red, the mammalian γ-subunit structure with three AMP molecules (yellow) and one ADP molecule (orange) bound in the center formed by CBS domains. **From Steinberg and Kemp, 2009.**

1.5.2 Mutations modulating AMPK activity

Commonly prescribed anti-diabetes drugs like metformin have been shown to activate AMPK (Zhou et al., 2001). Moreover, specific AMP-analogs like 5-aminoimadazole-4-carboximide-1-β-4-ribofuranoside (AICAR) are available. However, AMPK activity is also directly and chronically activated by mutations that affect the protein structure of the regulatory or catalytic residues (Merrill et al., 1997, Musi et al., 2002, Viollet and Foretz, 2016). Several of these mutations occur naturally, while others have been introduced artificially into animal models to study the function of AMPK in the regulation of tissue-specific, as well as whole-body energy metabolism. In humans, an activating mutation within the gene of the heart-specific γ2 isoform has been linked to the hereditary Wolff-Parkinson-White syndrome, which is associated with increased cardiac glycogen storage and tachycardia (Gollob et al., 2001). However, the best described AMPK mutation occurs naturally in Hampshire pigs and causes a vast accumulation of glycogen in skeletal muscle, as well as elevated levels of citrate synthase activity associated with an increased oxidative capacity (Milan et al., 2000, Granlund et al., 2010). This gain-of-function point mutation (AMPKγ3^{R225Q}) mainly affects glycolytic skeletal muscle, as the γ3-subunit is primarily expressed in glycolytic fibers (Mahlapuu et al., 2004). When this mutation was introduced into mice, it was furthermore shown that it protects against

intramuscular triglyceride accumulation and stimulates mitochondrial biogenesis. Moreover, this mutation protects against diet-induced insulin resistance, underlining not only the impact of tissue-specific AMPK activity on bioenergetics, but also the important role skeletal muscle plays in the regulation of whole-body metabolism in general (Barnes et al., 2004).

A similar mutation, also causing elevated glycogen levels and reduced intramuscular lipids, was described in the highly conserved human *PRKAG3* gene (AMPKγ3^{R225W}) (Costford et al., 2007). Gain-of-function mutations of the γ-subunit often render the complex constitutively active, as the altered protein structure bypasses the need for AMP to activate the enzyme complex. Although the role of AMPKγ3 has been studied extensively in the context of glycolytic skeletal muscle, the impact of alterations of AMPK activity in skeletal muscle via the mutation of the γ-subunit on whole-body glucose and lipid metabolism is not well understood. Additionally, the role the other γ-isoforms, especially AMPKγ1, play in metabolic regulation remains unclear. AMPKγ1 appears to be the most common γ-subunit in AMPK complexes in skeletal muscle, even though it is not as highly expressed as γ3 (Mahlapuu et al., 2004, Wojtaszewski et al., 2005).

The regulatory network of AMPK is very complex and in addition to gain-of-function mutations of the enzyme, loss of one or several subunit isoforms can have metabolic effects. In skeletal muscle, the AMPKγ isoforms appear to functionally compensate, to some extent, for the loss of one another. Moreover, AMPKγ3 knockout (KO) mice only show mild impairments in glycogen storage and glycogen resynthesis in EDL muscle, with preserved exercise capacity (Barnes et al., 2004). In contrast, the global deletion of both α-isoforms is embryonically lethal (Viollet et al., 2009). While the deletion of only AMPKα1 results in reduced lean mass and adiposity of mice (Daval et al., 2005, Fu et al., 2013), the deletion of AMPKα2 causes a diabetic phenotype, with insulin resistance and impaired glucose tolerance (Viollet et al., 2003). This sheds light on the interaction of AMPKα1 and α2, which are both expressed in tissues regulating glucose homeostasis such as liver and skeletal muscle.

Only in recent years, the role of the β-subunit of AMPK has been investigated more thoroughly since this subunit was previously believed to only fulfill a scaffolding function within the complex. Interestingly, AMPKβ1 KO mice show a lean phenotype, with a 90% reduction of AMPK activity in liver and no change in skeletal muscle or heart (Dzamko et al., 2010). Conversely, AMPKβ2 KO mice show reduced AMPK activity in skeletal muscle, with impaired exercise capacity and increased susceptibility to diet-induced obesity (Steinberg et al., 2010). In both models, the expression of the AMPKα subunits is greatly reduced in the affected tissues.

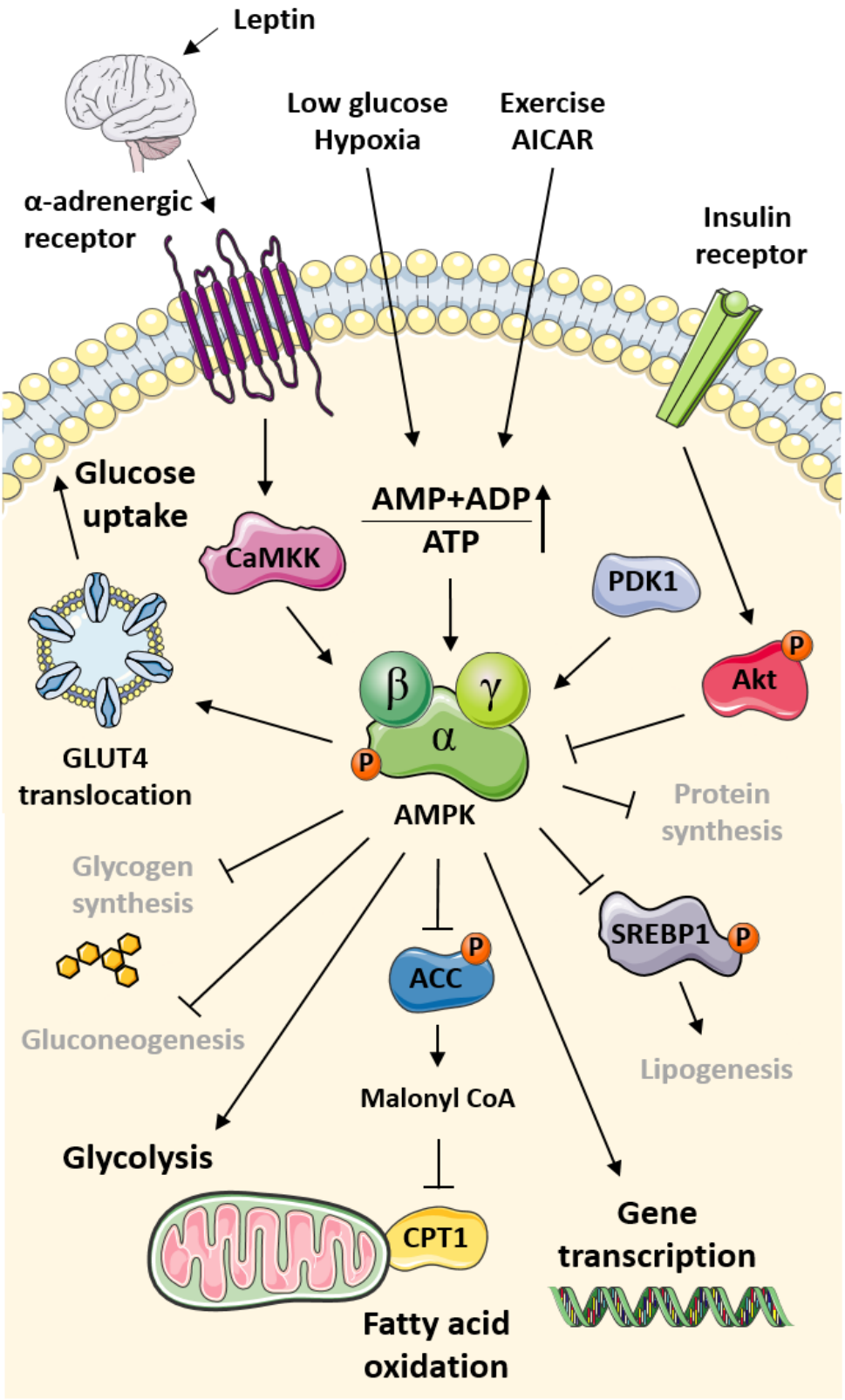

Figure 3: Regulation of metabolism by AMPK. Various stimuli activate AMPK and induce catabolic signals to generate more ATP, while inhibiting anabolic energy-storing pathways.

1.6 DIACYLGLYCEROL KINASES

Diacylglycerol kinases (DGKs) are a family of enzymes that drive the conversion of diacylglycerol (DAG) to phosphatidic acid (PA). Both lipids have distinct signaling, as well as metabolic functions. Thus, DGKs provide a link between lipid metabolism and signaling (Fig. 4). Ten different DGK isoforms, in addition to some alternatively spliced variants, have been described in mammals (α, β, γ, δ, ε, ζ, η, θ, ι and κ). DGKs are stimulated upon PI 3-kinase pathway activation, as well as by other upstream regulators such as Src kinase or PKC, in an isoform-specific manner (Sakane et al., 2007). All isoforms have at least two common cysteine-rich C1 domains that are associated with DAG binding and a catalytic domain (Imai et al., 2005, Shulga et al., 2011). Based on other structural motifs and homology domains, these ten isoforms can be further divided into five subtypes, which possibly carry out distinct biological functions. Several different DGK isoforms can be found to be co-expressed in most cells and tissues (Crotty et al., 2006), with tissue-specific expression profiles for example in skeletal muscle, where DGKα and ζ have been found to be most abundantly expressed in EDL and DGKδ in soleus muscle of mice (Manneras-Holm et al., 2015).

Besides the tissue-specificity of DGK isoforms, the functionality has been investigated in regard to the subcellular localization. Several isoforms were found to shuttle in and out the nucleus, while others are stimulus-dependent or permanently associated with the plasma membrane (Kobayashi et al., 2007). PA, the product of the DAG conversion that is catalyzed by DGK, is involved in the regulation of cellular events such as proliferation, cell survival, vesicle trafficking, as well as the phosphatidylinositol (PI) cycle involving lipid movement between cell organelles (Cazzolli et al., 2006). DGKε appears to be the only isoform that shows substrate specificity for DAG with arachidonoyl acyl chains in the sn-2 position and is thus directly involved in the PI cycle, as all intermediates of this cycle are rich in arachidonoyl groups (Tang et al., 1996). Studies in knockout mouse models have established that DGKε is involved in the regulation of seizure susceptibility and that DGKα and ζ play important roles in the regulation of the function of lymphocytes linking DGKs to cancer (Olenchock et al., 2006, Rodriguez de Turco et al., 2001). All DGK isoforms are found in the central nervous system and directly associated with neuronal and glial function (Goto and Kondo, 2004).

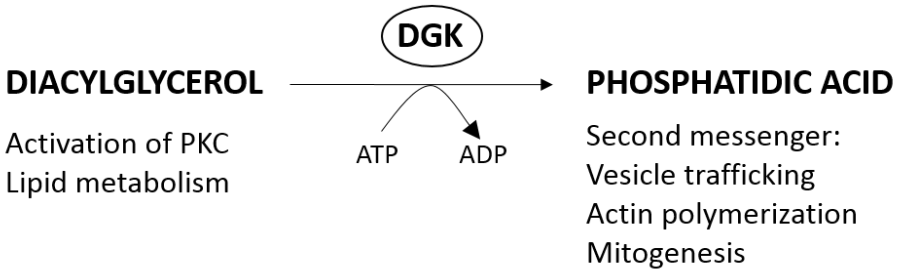

Figure 4: Role of diacylglycerol kinase in lipid signaling and metabolism. DGK drives the phosphorylation of the free hydroxyl group of DAG to produce phosphatidic acid.

1.6.1 Role of DGKs in metabolism

In metabolically active tissues, DAG levels and DGK activity have been directly linked to metabolic health as insulin resistance is associated with increased intracellular DAG levels (Kraegen et al., 2006). A reduced expression of DGKδ was found in skeletal muscle from type 2 diabetic subjects and DGKδ haploinsufficiency in mice has been shown to be sufficient to reduce peripheral insulin sensitivity and promote age-dependent obesity (Chibalin et al., 2008). Low-intensity exercise in type 2 diabetic patients improves clinical parameters such as HOMA-IR, a measurement of insulin resistance, and also increases DGKδ expression in skeletal muscle, underlining the tie of DGKs to metabolic homeostasis (Fritz et al., 2006). Diminishing expression levels of DGKα and DGKγ in pancreatic β-cells attenuates the secretion of insulin while the global loss of DGKζ protects against diet-induced peripheral insulin resistance (Kurohane Kaneko et al., 2013, Benziane et al., 2017). In addition, the abundance of DGKε mRNA was found to be reduced in EDL muscle and epididymal adipose tissue from obese insulin resistant *ob/ob* mice (Manneras-Holm et al., 2015). However, the role of DGKε, with its distinct biological function regarding the conversion of certain lipid species, in the regulation of skeletal muscle glucose and lipid metabolism as well as in whole-body physiology remains unclear.

2 AIMS

The importance of the regulation of whole-body energy homeostasis by skeletal muscle is eminent in light of the detrimental effects of skeletal muscle insulin resistance and reduced metabolic flexibility on the whole organism. The roles of two kinases constituting regulatory hubs in this system, DGKε and AMPK, and their effect on glucose and lipid metabolism in skeletal muscle and the whole body are incompletely understood. Characterizing the function of these enzymes in the regulation of metabolism may aid the understanding of causative mechanisms and potential prevention strategies for the treatment of metabolic diseases. In addition, gaining insights into the plethora of cellular changes in skeletal muscle concomitant with the increased exposure to glucose and fatty acids in obesity could help identify novel treatment targets for insulin resistance.

The aims of this thesis are therefore to:

- Elucidate the contribution of DGKε to the regulation of energy homeostasis on the whole-body level and in skeletal muscle in relation to insulin resistance and obesity.

- Characterize the role of the regulatory skeletal muscle AMPK subunits γ1 and γ3 in the maintenance and regulation of whole-body glucose and lipid metabolism and the effects skeletal muscle AMPK activation has on other peripheral tissues.

- Identify changes in the skeletal muscle proteome of leptin-deficient and insulin resistant *ob/ob* mice to further characterize metabolic adaptations to obesity that could be targeted with pharmacological approaches.

3 EXPERIMENTAL CONSIDERATIONS

This section focuses on specialized *in vivo* and *in vitro* methods that are not routinely used in general biomedical laboratories (such as immunoblotting or quantitative PCR) but that were used in the animal studies presented in this thesis.

3.1 ANIMALS

All mice used in the studies presented were bred on a C57BL/6J background and, unless indicated otherwise, had free access to standard rodent chow and water and were housed in group cages in a temperature controlled environment (24ºC) with a 12 hours light/dark cycle. All experiments were approved by the regional animal ethical committee, Stockholm, Sweden. The models used in paper I-IV are presented in Table 1 and the generation of the model used in paper II is described in more detail below.

Table 1

Study	Animal model	Details
Paper I	DGKε knockout	Whole-body deletion of exon 1 of DGKε (Rodriguez de Turco et al., 2001), the only diacylglycerol kinase with a hydrophobic segment (Decaffmeyer et al., 2008).
Paper II	AMPKγ1^{H151R}	MLC1-Cre-induced expression of an activating AMPKγ1^{H151R} transgene specifically in skeletal muscle.
Paper III	AMPKγ3^{R225Q}	Skeletal muscle-specific MLC1-promoter driven expression of an activating AMPKγ3^{R225Q} transgene (Barnes et al., 2004).
Paper IV	*ob/ob*	Complete lack of leptin due to a mutation in the *obese* gene (Ingalls et al., 1950).

To generate the skeletal muscle-specific AMPKγ1^{H151R} mouse line, mice expressing Cre recombinase under the myosin light chain (MLC1) promotor (kindly provided by Steven Burden, New York University Medical Center, NY, USA) were mated with mice carrying the Cre-inducible transgene AMPKγ1^{H151R}. The transgene is under the control of the β-actin promotor and contains the mutated human *PRKAG1* gene sequence located downstream of a stop sequence that is flanked by two loxP sites. This allows the specific expression of the transgene in skeletal muscle tissue due to the Cre-mediated excision of the stop sequence.

Histidine 151 in the human protein sequence refers to the previously described murine activating mutation of H150 in the CBS2 domain of the AMP-binding site of AMPKγ1 (Minokoshi et al., 2004). In paper II, we studied female and male floxed (fl/fl) AMPKγ1^{H151R} MLC1-Cre mice and wildtype littermates bred on mixed C57BL/6J background.

3.2 *IN VIVO* TECHNIQUES

3.2.1 Body composition

EchoMRI-100 (EchoMRI LLC, Houston, TX, USA) scanning allows for the rapid assessment of body composition of conscious animals based on nuclear magnetic resonance (NMR) and gives measures of total body fat, lean mass, free water and total body water.

3.2.2 Glucose tolerance

For all glucose tolerance tests presented in this thesis, mice were fasted for 4 hours in single cages and thereafter 2 g/kg glucose was administered intraperitoneally. Blood glucose was measured in tail tip blood at 0, 15, 30, 60 and 120 min (OneTouch Ultra 2 glucose meter, Lifescan, Milpitas, CA, USA) following the injection. Serum insulin concentration was measured at 0 and 15 min using an ELISA with mouse insulin as a standard (Crystal Chem., Chicago, IL, USA). This *in vivo* procedure allows for the assessment of glucose clearance capacity from the blood and can give an indication of general insulin sensitivity.

3.2.3 Hyperinsulinemic-euglycemic clamp

Glucose clamp techniques are the gold standard for measuring glucose utilization and insulin sensitivity *in vivo* (DeFronzo et al., 1979) and we employed the hyperinsulinemic-euglycemic clamp technique in conscious mice (Ayala et al., 2011). The assessment of peripheral insulin sensitivity is based on the quantification of glucose tracer clearance from the blood representing the general glucose turnover in combination with the quantification of the administered glucose required to maintain constant glycemia after insulin-mediated inhibition of hepatic glucose production and stimulation of peripheral glucose uptake.

In paper II, jugular vein catheterization was performed on mice under isoflurane anesthesia with carprofen analgesic treatment on the day of surgery and one day after. Animals were left to recover and monitored for at least 4 days in single cages. Using a constant jugular vein infusion of [3-^{3}H] glucose (2.5 µCi bolus and a flow rate of 0.04 µCi/min), glucose turnover rate was measured in the basal state and under hyperinsulinemic-euglycemic clamp conditions in 4-hour-fasted mice. Basal glucose utilization and hepatic glucose production was assessed 50-70 min after the start of the tracer infusion right before the insulin infusion was started. The clamp was started with a priming dose of insulin (17.5 mU/kg; Actrapid, Novo Nordisk, Bagsvaerd, Denmark) followed by a constant infusion of insulin at a rate of 1.75 mU/kg/min. At steady state (~85 min after the start of the insulin infusion), blood samples were collected and whole-body glucose utilization was measured. Hepatic glucose production was calculated by subtracting the glucose infusion rate from the glucose utilization. Additional blood samples

were taken at basal and clamped state to determine serum insulin concentrations by ELISA. Animals were euthanized with an overdose of sodium pentobarbital.

3.2.4 Whole-body energy homeostasis (metabolic cages)

Housing animals in metabolic cages allows for the simultaneous measurement of food intake, water consumption, movement, heat production, O_2 consumption (VO_2), CO_2 production (VCO_2), and the calculation of the respiratory exchange ratio (RER) over hours or days. This analysis gives insight into whole-body metabolism and changes caused by mutations or dietary interventions. The RER indicates whether carbohydrates or lipids are primarily metabolized, with an RER close to 1 indicating carbohydrate utilization and values close to 0.7 indicating lipid oxidation. In paper I and II, mice were acclimated for at least 24 hours in single cages and subsequently monitored for up to 2 days in the Oxymax Lab Animal Monitoring System (CLAMS, Columbus Instruments, Columbus, OH, USA) with *ad libitum* access to food and one night without access to food (12-hour fasting, only in paper II). In this system, the gas exchange was recorded every 20 min by sampling the air from the individual cages and passing it through sensors to determine the O_2 and CO_2 content. Spontaneous locomotor (ambulatory) activity was measured by consecutive light beam breaks of adjacent beams on the X, Y and Z axes. An alternative metabolic cages system for small rodents is the TSE PhenoMaster home cage system (TSE Systems, Germany) that also allows for the adjustment of the light/dark cycle and the temperature as the cages are inside cabinets to control the environment beyond the gas exchange.

3.2.5 Fatty acid oxidation

To assess the whole-body fatty acid oxidation rate in conscious mice as described in paper III, chow and high-fat diet fed male AMPK$\gamma3^{R225Q}$ and wildtype littermates were catheterized as described for the hyperinsulinemic-euglycemic clamp above. Prior to the experiment, mice were fasted for 2 hours and body weight was recorded, glycemia measured and fasted blood samples were collected. Blood samples were subjected to centrifugation for 6 min at 10,000g (4°C) and plasma stored at -80°C. Infusate per mouse was prepared with 10^7 DPM of [9,10-^{3}H(N)]-Palmitic Acid (NET043001MC, PerkinElmer, CA, USA) and 10^7 DPM of non-oxidizable [1-^{14}C]-2-bromopalmitic acid (MC 451, Moravek Inc., CA, USA) dried under a nitrogen steam and reconstituted in 100 µl of saline containing 1.2% BSA and 0.15 mM palmitate. Before the start of the infusion of the tracer (t=0), the catheter was flushed with saline to ensure patency and the infusion rate set to 20 µl/min. Blood samples (15 µl) were collected from the cut tail tip using heparinized capillaries at t=0, 1, 3, 5, 7, 9 and 12 min and directly frozen in liquid N_2. At t=5 min, the pump (Univentor, Malta) was disconnected and the catheter was flushed again with saline. After the last blood sample collection, mice were euthanized via an injection of pentobarbital sodium into the jugular vein (t=16 min) and skeletal muscle (TA, EDL and soleus), perigonadal white adipose tissue, liver and heart were dissected, cleaned from blood and quickly frozen in liquid N_2. This time point was selected to ensure that the last blood sample collection could be finished for all animals and still ensure the same euthanasia time

point allowing the comparison of the analyzed tissues and the metabolites therein. In the case of pre-treatment, mice were injected with 5 mg/kg (+)-etomoxir sodium salt hydrate (Sigma Aldrich, Germany), an inhibitor of CPT1, dissolved in saline into the jugular vein 15 min prior to the tracer infusion.

3.3 *IN VITRO* TECHNIQUES

3.3.1 Glucose transport in isolated skeletal muscle

In paper I, glucose uptake was assessed *in vitro* in isolated skeletal muscle from 4-hour-fasted DGKε KO and wildtype mice. Mice were anaesthetized (2.5% Avertin; 0.02 ml/gram body weight) and EDL and soleus muscles were removed with intact tendons. Incubation media was prepared from a stock solution of Krebs-Henseleit bicarbonate buffer (KHB) supplemented with 5 mM HEPES and 0.1% bovine serum albumin (RIA grade) and continuously gassed with 95% O_2/5% CO_2 to maintain the muscles in a physiological environment. Following the dissection, muscles were incubated to recover at 30°C for 30 min in KHB containing 5 mM glucose and 15 mM mannitol. Muscles were then incubated for 30 min in KHB with the same supplements as the recovery buffer but in the additional absence or presence of a submaximal dose of insulin (0.36 nM) and subsequently rinsed for 10 min in KHB containing 20 mM mannitol as well as insulin as before, but without glucose. Thereafter, muscles were incubated for 20 min in the absence or presence of insulin in KHB buffer containing 19 mM [^{14}C] mannitol (0.7 mCi/ml) and 1 mM (^{3}H) 2-deoxyglucose (2.5 mCi/ml), allowing for the quantification of insulin-stimulated glucose uptake by assessing the intracellular accumulation of [^{3}H] 2-deoxyglucose-6-phosphate in the muscles (Wallberg-Henriksson, 1987, Hansen et al., 1994).

3.3.2 Lipid extraction of radioactive palmitate

Several solvent-based systems are commonly used to extracts lipids from biological samples but especially the hexane-isopropanol system (Hara and Radin, 1978) was found to be suitable for extracting hydrophobic lipids, such as free fatty acids, triglycerides and cholesterol esters (Reis et al., 2013). In the blood and tissues samples collected during the *in vivo* fatty acid oxidation experiments described in paper III, the infused radioactive lipids were separated from the ß-oxidation by-product $^{3}H_2O$ (in the aqueous phase) as described (Massart et al., 2014). Samples were rotated overnight at room temperature with an addition of 300 µl isopropanol/0.1% acetic acid allowing the diffusion of the lipids into the solvent. Samples were then rotated for an additional 10 min after an addition of 600 µl hexane and 150 µl 1 M KCl which improves the removal of non-lipid contaminants (Hara and Radin, 1978). The phases were allowed to separate and the upper organic phase containing the lipids was collected, vacuum-dried for 1 hour and reconstituted in 50 µl methanol:chloroform (1:1) before being transferred into scintillation vials. The lower phase (aqueous phase) was treated with 300 µl 1 M NaOH and shaken for 30 min at 50°C to dissolve cell debris. After neutralization with 300 µl 1 M HCl, the samples were transferred into scintillation vials and radioactivity was measured (1414 Win Spectral Liquid Scintillation Counter, Wallac/PerkinElmer, Turku, Finland).

3.3.3 Proteomics of skeletal muscle tissue

The identification of proteins and peptides with proteomics techniques is critically dependent on the sample preparation. As described in paper IV, the protein lysates from quadriceps muscle of male *ob/ob* mice and mouse myoblast C2C12 cells were processed with a multiple enzymes digestion with filter-aided sample preparation (MED-FASP) using the endoproteinase LysC and trypsin. LysC supplements the trypsin-mediated proteolysis, especially of tightly folded proteins, ensuring full protein cleavage (Swaney et al., 2010). The peptides were purified using C_{18} Stage tips that employ the principle of solid phase extraction with the analyte passing through a Teflon mesh with reversed-phase C_{18}-coated silica beads binding non-polar particles (Rappsilber et al., 2003). As part of the LC-MS instrumentation, the Easy nano-flow high-performance liquid chromatography (HPLC) system (Thermo Fisher Scientific, Waltham, MA, USA) was used for the separation of the peptides. The samples (2 µg) were loaded with buffer A (0.5% formic acid) onto 50 cm C_{18} columns and eluted with a 280 min linear gradient from 2-30% buffer B (80% acetonitrile, 0.5% formic acid). Mass spectra were acquired in the Orbitrap analyzer with tandem mass spectrometry (Thermo Fisher Scientific). This set-up allows for the detection of the ionized molecules during the first stage and the measurement of the mass/charge (m/z) ratio of the fragmented molecules following high-energy collision dissociation (HCD) in the second stage (Fig. 5). The analysis of HCD peptide fragments facilitates the identification of proteins and peptides, as the breaking points of individual molecules are predictable or known, and therefore allows for easier matching of the results with protein databases. We furthermore matched the data from skeletal muscle to data from cultured differentiated mouse C2C12 myoblasts, which improved the peptide identification and increased the depth of the analyzed proteome by ~25%.

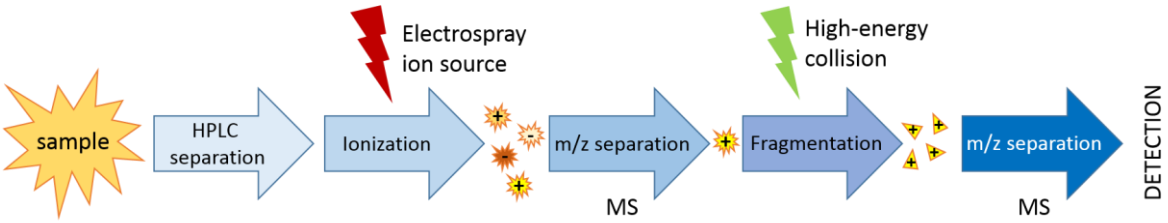

Figure 5: Liquid chromatography-mass spectrometry workflow. The peptides are separated on HPLC columns and mass spectra of the ionized peptides as well the peptide fragments are acquired using tandem mass spectrometry.

3.3.4 Lipidomics of skeletal muscle tissue

Similar to the assessment of the proteome of a cell, tissue or organism, the metabolome can be investigated using mass spectrometry. As a subset of metabolites, the different lipid species (Fig. 6, (Roberts et al., 2008)) in a sample can be identified and ratios between them can be established to give insight into metabolic alterations directly linked to lipid utilization or molecular signaling. In paper I, lipidomic analysis was performed on gastrocnemius muscle of 4-hour-fasted high-fat diet fed DGKε KO and wildtype mice. The tissue samples were mechanically disrupted and lipids were extracted using chloroform and methanol (Folch et al.,

1957). Following centrifugation, the lipid-containing bottom phase was treated with isopropyl alcohol:methanol and 20 mM of ammonium acetate before the sample was administered into the infusion stream of a 5600 QQ Tof mass spectrometer (Sciex, Framingham, MA, USA) in electrospray mode at a flow rate of 20 ml/min. The sample was spiked with a series of internal saturated lipid standards which were used for normalization, resulting in a height ratio output. The internal standards used were C15:0 DAG, D5 tripalmitin, C14:0 phosphatidylcholines (PCs), C17:0 sphingomyelin, C17:0 ceramide, C15:0 lysophosphatidylcholines, and C15:0 phosphatidylethanolamine (PE).

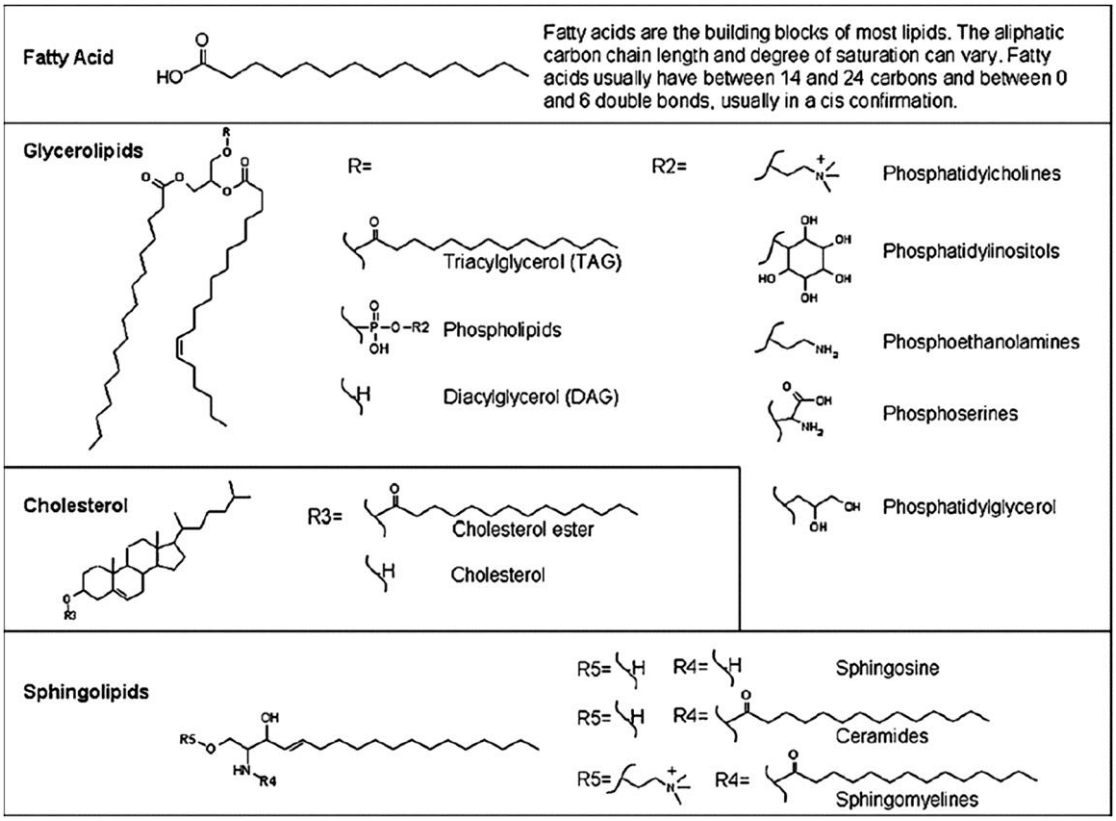

Figure 6: Summary of the structural diversity of the most commonly analyzed lipid species. From Roberts et al., "A matter of fat: An introduction to lipidomic profiling methods", 2008. Used with permission from Elsevier.

3.4 STATISTICS

3.4.1 Paper I, II and III

All data are presented as mean ± SEM. Differences were determined by Student's *t*-test or two-way ANOVA where applicable followed by Bonferroni's post hoc test. Differences were considered statistically significant at $p<0.05$. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software Inc., CA).

3.4.2 Paper IV

For the global bioinformatics analysis, two sample *t*-tests were performed on Lean and Ob (*ob/ob*) groups with FDR=0.05 and S0=0.1. Hierarchical clustering of significantly different proteins was performed after Z-score normalization. Fisher's exact tests were performed on

particular clusters, testing for enrichment or depletion of any annotation term in the cluster compared to the whole matrix. For the assessment of total protein abundance in the proteomics dataset and the Western blot analysis, differences between Lean and Ob were determined by Student's *t*-test, with significance at $p<0.05$. Results are presented as median ± SD for the proteomics data and as mean ± SD for the Western blots.

4 SUMMARY OF THE MAIN FINDINGS

Paper I

Whole-body ablation of DGKε in mice elevated saturated and unsaturated DAG species in skeletal muscle without affecting liver triglyceride levels, body weight or body composition. Whole-body RER was decreased under high-fat fed conditions, indicating increased reliance on lipids as fuel source, yet whole-body glucose tolerance was increased albeit unchanged insulin-stimulated skeletal muscle glucose transport. These results support the notion that DGKε plays a role in modulating lipid metabolism in skeletal muscle affecting whole-body energy homeostasis.

Paper II

The skeletal muscle-specific overexpression of AMPKγ1^{H151R} altered whole-body metabolic homeostasis with increased RER and insulin sensitivity, as well as altered energy expenditure. Several sex-specific effects were noted such as the reduction of perigonadal white adipose tissue mass and serum leptin in female AMPKγ1^{H151R} mice. Together these findings suggest that the activation of AMPKγ1 specifically in skeletal muscle alters metabolic homeostasis favoring glucose utilization and may protect against the development of insulin resistance.

Paper III

The assessment of whole-body lipid oxidation with an *in vivo* assay relying on the intravenous administration of [9,10-^{3}H(N)]-palmitic acid combined with the non-β-oxidizable palmitate analogue [1-^{14}C]-2-bromopalmitic acid showed no differences between skeletal muscle-transgenic AMPKγ3^{R225Q} and wildtype mice. However, the suppression of mitochondrial lipid oxidation by the CPT1 inhibitor etomoxir and an overall increase of whole-body lipid oxidation under high-fat fed conditions were detected.

Paper IV

Analyzing skeletal muscle from obese and leptin-deficient *ob/ob* mice with an efficient state-of-the-art proteomics workflow led to the identification of over 6,000 proteins of which 118 were differentially abundant in comparison to lean mice. Enzymes involved in lipid metabolism, proteins characteristic for oxidative type I fibers and mitochondrial, as well as peroxisomal, proteins were upregulated in quadriceps muscle from *ob/ob* mice together indicating increased fatty acid oxidation.

5 RESULTS AND DISCUSSION

5.1 ROLE OF DGK IN THE REGULATION OF WHOLE-BODY METABOLISM

DGKε is a member of a family of diacylglycerol kinases that catalyze the conversion of diacylglycerol into phosphatidic acid and thereby constitute a regulatory hub in DAG signaling, as well as lipid metabolism. DGKε is the only DGK isoform showing substrate specificity for DAGs with arachidonoyl acyl chains and plays a distinct role in enriching inositol phospholipids with unsaturated and saturated fatty acids. In paper I, we investigated the effect of the whole-body deletion of DGKε in mice on the regulation of energy homeostasis in relation to diet-induced obesity and insulin resistance with special focus on skeletal muscle.

5.1.1 DGKε deficiency increases DAG species, glucose tolerance and lipid oxidation

We provide evidence that the loss of DGKε alters the lipid profile of skeletal muscle as seen in the accumulation of unsaturated and saturated DAGs, as well as short-acyl-chain triglycerides (Fig. 7). This was expected and in line with previous research focusing on the effect the loss of other DGK isoforms, such as DGKδ, has on metabolic homeostasis (Chibalin et al., 2008). Paradoxically, the elevation of DAGs seen in high-fat fed DGKε KO mice was associated with increased reliance on lipids as fuel source as well as overall enhanced glucose tolerance (Fig. 8). This indicates that although DGKε expression was found to be reduced in skeletal muscle and adipose tissue of obese and insulin resistant *ob/ob* mice (Manneras-Holm et al., 2015), the complete loss of DGKε, in turn, does not appear to contribute to the metabolic disturbances associated with obesity and insulin resistance. However, our study does not address the effect a transient reduction of DGKε expression, possibly occurring with increasing obesity may have. Moreover, we cannot exclude the possibility that an acute deletion of DGKε may also affect metabolic parameters differentially. The comparison with a model of acute DGKε deletion via a tamoxifen-dependent knockout would give further insight into the role of DGKε in the development of metabolic diseases.

High-fat fed DGKε KO mice exhibited increased abundance of mitochondrial enzymes in skeletal muscle concomitant with the changes in whole-body glucose and lipid metabolism. However, insulin sensitivity was unchanged in isolated skeletal muscle pointing towards the involvement of other tissues regulating whole-body metabolism. To further investigate the effect of DGKε deficiency on glucose metabolism, we performed a preliminary experiment to assess whole-body glucose uptake using a hyperinsulinemic-euglycemic clamp in high-fat fed male DGKε KO mice (Mannerås-Holm *et al.*, unpublished). Hepatic glucose production was unchanged in DGKε KO mice, indicating that tissues other than the liver, possibly adipose tissue, may be involved. Additional studies focusing on the glucose uptake and regulation of metabolic homeostasis by adipose tissue, also with regard to lipolytic activity, in DGKε KO mice may answer these remaining questions. Together, the data presented in this study suggest that DGKε deficiency leads to an overall increase in fatty acid oxidation capacity and lipid

turnover, but the characterization of the specific effect on *de novo* lipid synthesis and glycerol metabolism, for example by using radioactively labelled substrates, requires further analyses.

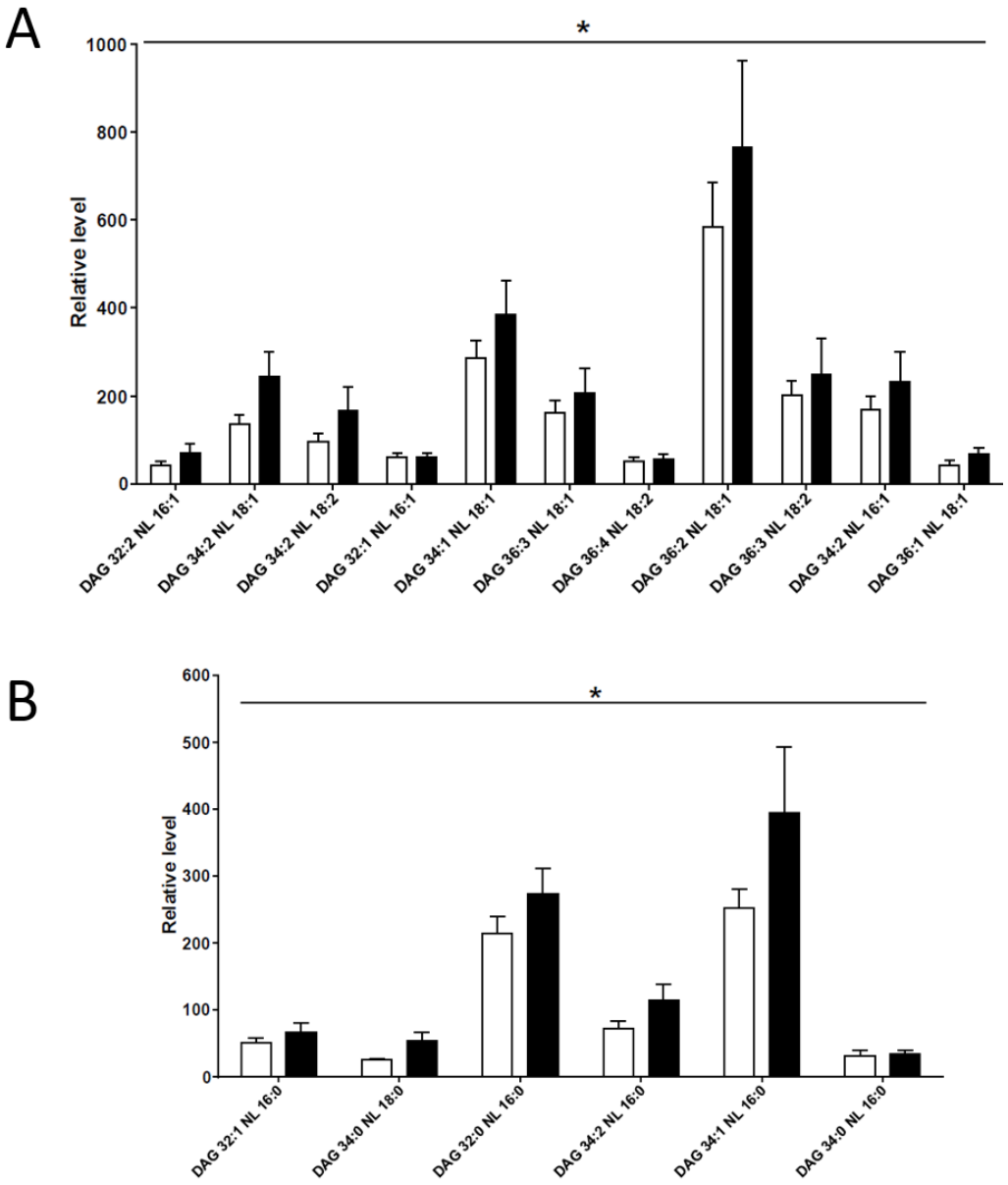

Figure 7: Impact of DGKε on DAG levels in skeletal muscle. Gastrocnemius muscle was obtained from high-fat fed WT mice (open bars) and DGKε KO mice (solid bars) for lipidomics analysis of unsaturated (A) and saturated (B) DAG species. *$p < 0.05$. **Refers to Fig. 1 in paper I.**

5.1.2 Skeletal muscle of DGKε deficient mice exhibits the "athlete's paradox"

The results of increased DAGs in skeletal muscle paired with elevated glucose tolerance are not consistent with the notion that skeletal muscle lipid accumulation is linked to insulin resistance. However, they reflect the "athlete's paradox" where high oxidative capacity in skeletal muscle of well-trained athletes is paired with the accumulation of intracellular lipids without negatively impacting insulin sensitivity (Goodpaster et al., 2001). The change of the activity of a DGK isoform associated with a distinct subcellular region may alter the subcellular localization and accessibility of certain lipids in lipid droplets and hence modulate lipid

metabolism and partitioning. Athletes receiving a continuous lipid infusion concurrent with a hyperinsulinemic-euglycemic clamp, to inhibit lipolysis, showed not only an accumulation of intramuscular lipids specifically in type I fibers, but also a redistribution of perilipin (PLIN) proteins to newly formed lipid droplets, which was not observed in sedentary subjects (Shepherd et al., 2017, Townsend and McKie, 2017). One of the PLIN isoforms found in these newly formed droplets was PLIN2, which is directly linked to triglyceride synthesis via its association with diacylglycerol acyltransferase 2 (DGAT2), the enzyme catalyzing the conversion of DAGs into triglycerides (McIntosh et al., 2012). This draws further attention to the regulation of lipid metabolism by lipid droplet biochemistry, and could possibly help explain the physiological effects seen in animal models with altered DGK activity.

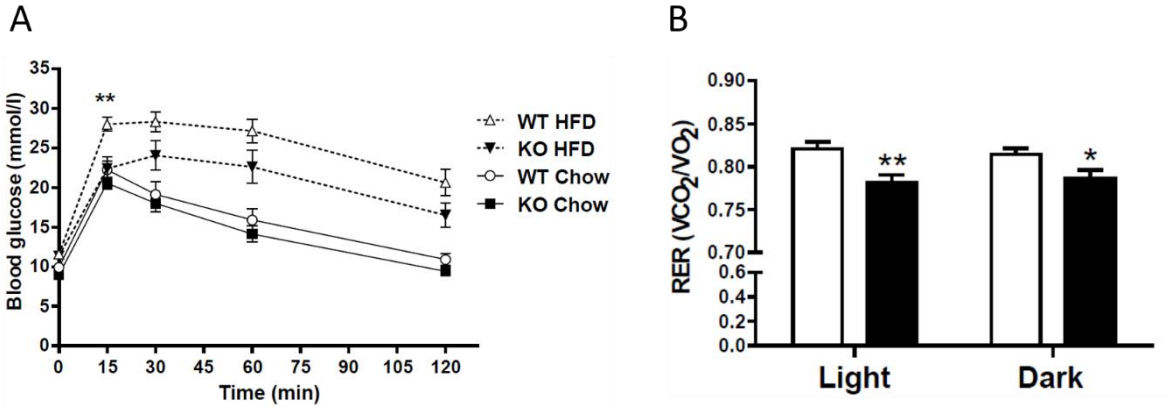

Figure 8: DGKε deficiency enhances glucose tolerance and alters energy homeostasis. Intraperitoneal GTT was performed in 4 h fasted high-fat fed WT mice (open triangle; n = 14) and DGKε KO mice (solid triangle; n = 11) as well as chow fed WT mice (open circle; n = 11) and DGKε KO mice (solid square; n = 12) (A). RER was assessed by indirect calorimetry in high-fat fed WT mice (open bar; n = 11) and DGKε KO mice (solid bar; n = 10). Results are presented as the mean ± SEM. *P < 0.05. **P < 0.01 WT versus KO mice (Student *t*-test). **Refers to Fig. 3/5 in paper I.**

5.1.3 Distinct metabolic changes following the loss of other DGK isoforms

The ablation of DGKζ, another isoform highly abundant in skeletal muscle, leads to modest growth retardation and reduced adiposity indicating a function of this DGK family member during growth and development. Similar to DGKε KO mice, high-fat fed DGKζ KO mice are protected against insulin resistance despite increased DAG content in skeletal muscle (Benziane et al., 2017). Conversely, the loss of DGKα causes mild insulin resistance in chow-fed mice, which is reversed after long-term high-fat feeding. Since DGKα, together with DGKζ, is the predominant DGK isoform found in T-cells, it plays a role in modulating inflammatory responses. Indeed, the expression of inflammatory markers was modestly increased in adipose tissue of short-term high-fat fed DGKα KO mice (Nascimento et al., 2017). Together, these studies suggest that the ablation of either DGKα, ε or ζ causes relatively mild metabolic alterations in comparison to the detrimental effects of DGKδ haploinsufficiency on insulin sensitivity and metabolic flexibility (Chibalin et al., 2008). Interestingly, concomitant with impaired lipid oxidation, AMPK activation and signaling was reduced in skeletal muscle of DGKδ heterozygous mice (Jiang et al., 2016). Further studies focusing on the tissue-specific role of DGKδ in skeletal muscle and adipose tissue are warranted to

investigate whether site-specific activation of DGKδ constitutes a target for the treatment of insulin resistance.

5.2 IMPACT OF ACTIVATING AMPKγ MUTATIONS IN SKELETAL MUSCLE ON METABOLISM

The γ-subunit of AMPK regulates the activity and function of the heterotrimeric enzyme complex that acts as an energy sensor and modulator of cellular metabolism to match the energy demand within the cell. In paper II, we investigated the role of AMPKγ1 in the regulation of skeletal muscle and whole-body metabolism by overexpressing the mutated isoform AMPKγ1^{H151R} specifically in skeletal muscle. In paper III, we assessed the impact of the skeletal muscle-specific expression of AMPKγ3^{R225Q} on whole-body lipid metabolism with an *in vivo* assay relying on the infusion and oxidation of radioactively labelled lipids.

5.2.1 AMPK activation in skeletal muscle increases glycogen storage and promotes whole-body carbohydrate metabolism

We show that the expression of AMPKγ1^{H151R} specifically in skeletal muscle improves whole-body insulin sensitivity and promotes glucose utilization while preserving the ability to switch between carbohydrates and lipids as fuel source (Fig. 9). Together with previously described models of mutations bypassing the need for AMP-binding to the AMPKγ subunit to activate the complex (Barnes et al., 2004, Barre et al., 2007), these data indicate that the main phenotypic effects described are probably not linked to particular γ-subunits, but most likely the result of a general activation of AMPK. Despite high levels of phosphorylated and hence inhibited glycogen synthase, all models of chronic AMPK activation show an accumulation of glycogen in skeletal muscle, arguably due to the overwriting stimulating effect increased glucose-6-phosphate concentration has on GS (Roach et al., 2012). Indeed, in skeletal muscle of pigs carrying an activating AMPK mutation (AMPKγ3^{R200Q}), glucose-6-phosphate was reported to induce GS activity to a greater extent than in wildtype pigs (Scheffler et al., 2016). Interestingly, glycogen accumulation was preserved in pigs with an additional mutation affecting the gene of the ryanodine receptor 1 (RyR1^{R615C}) that reportedly blocks AMPK-induced GLUT4 expression via elevating cytosolic calcium (Park et al., 2009). The authors connect this to an increased expression of UDP-glucose phosphorylase 2 in skeletal muscle of pigs carrying the AMPKγ3^{R200Q} mutation that possibly shuttles more glucose towards glycogen synthesis (Scheffler et al., 2016). However, we report increased glucose oxidation in AMPKγ1^{H151R} transgenic mice, as indicated by a higher RER, despite robust inhibition of ACC in skeletal muscle and hence stimulation of fatty acid oxidation (Fig. 9). We conclude that these molecular changes, allowing for an elevated rate of glucose and lipid oxidation, support increased metabolic flexibility in a system constantly challenged to meet a high energy demand.

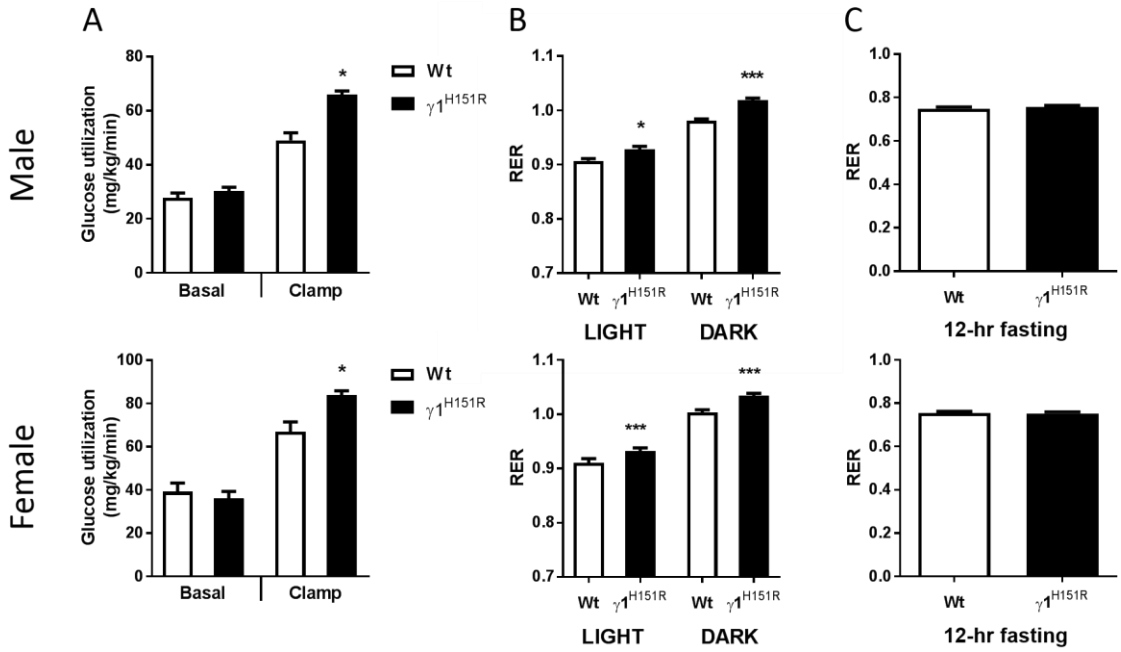

Figure 9: Skeletal muscle AMPKγ1^{H151R} expression promotes whole-body glucose utilization and preserves metabolic flexibility. Whole-body glucose utilization was assessed in conscious 17-week-old male and female Wt (open bars) and AMPKγ^{1H151R} transgenic mice (black bars) at basal and clamped states (n=3-8) (A). Respiratory exchange ratio (RER) was measured in male and female Wt and AMPKγ1^{H151R} transgenic mice (16 –22 weeks of age) for 2 consecutive days (B) and one overnight (12 hours) fast (n=6-8) (C). Results are means ± SEM. *P ≤ 0.05 and ***P ≤ 0.001 vs. respective Wt mice. **Refers to Fig. 9/6 of paper II.**

5.2.2 Sex-specific metabolic effects caused by the expression of AMPKγ1^{H151R} in skeletal muscle

Sex-specific differences are frequently observed in physiological, pharmaceutical and behavioral studies in humans and animals. The further elucidation of metabolic differences and the underlying differences is important for the improvement of treatment strategies for wide-spread diseases like obesity and diabetes in both men and women. Here we are the first to describe sex-specific effects of skeletal muscle-specific AMPK activation. Although total body weight was unchanged in both male and female AMPKγ1^{H151R} mice, males showed increased food intake while female mice had smaller perigonadal adipose tissue depots and reduced serum leptin and insulin levels (Fig. 10). Additionally, UCP1 mRNA in BAT and β3-adrenergic receptor mRNA in WAT was elevated in female AMPKγ1^{H151R} mice suggesting a direct connection between skeletal muscle AMPK activity and the regulation of adipose tissue metabolism. Skeletal muscle of men and women responds differently to exercise as submaximal exercise strongly induces the phosphorylation of AMPKα in skeletal muscle of men, but not in women (Roepstorff et al., 2006). This difference may be due to a better maintenance of the cellular energy balance in skeletal muscle of women. In addition, a higher percentage of myosin heavy chain I fibers was found in skeletal muscle of women compared to men, possibly explaining the higher rate of lipid oxidation observed during exercise in women (Guadalupe-Grau et al., 2016). At rest, however, the female mice in our study predominantly oxidized glucose similarly to male mice. These findings indicate that the activity of AMPK in skeletal muscle mediates sex-specific effects on other tissues and the whole body.

A direct binding of the estrogen receptor to AMPKα, resulting in the activation of the enzyme complex, was reported from cell-based studies (Lipovka et al., 2015). By utilizing ovariectomized mice lacking estrogen, the extent to which this mode of activation contributes to whole-body metabolic regulation in AMPKγ1^{H151R} mutant mice may be examined. Additionally, quantification of serum adipokines such as adiponectin, which is inversely correlated to obesity and insulin resistance, could give further insight into the AMPK-related connection between skeletal muscle and adipose tissue metabolism observed in AMPKγ1^{H151R} mice. Although women have higher plasma adiponectin, an association with leg glucose uptake and AMPK phosphorylation in skeletal muscle is only evident in men (Hoeg et al., 2013). This has again been linked to the sex-specific differences in skeletal muscle fibers as women have relatively less adiponectin receptor-expressing type II fibers (Bauche et al., 2007). In summary, the phenotypic effects observed in female AMPKγ1^{H151R} mice that go beyond those seen in male mice are possibly associated with a greater impact of chronic AMPK activation in skeletal muscle of female versus male mice. This may be a plausible explanation given that female mice usually exhibit only moderate changes of AMPK activity due to a tighter regulation of energy balance. The effect of skeletal muscle-specific AMPKγ1^{H151R} expression on gene expression in different adipose tissue depots is contradictory to the reduced energy expenditure detected in these mice. Thus, characterization of the primary metabolic link or secondary connection of these tissues via adipokines or myokines warrants further studies to elucidate the underlying mechanisms.

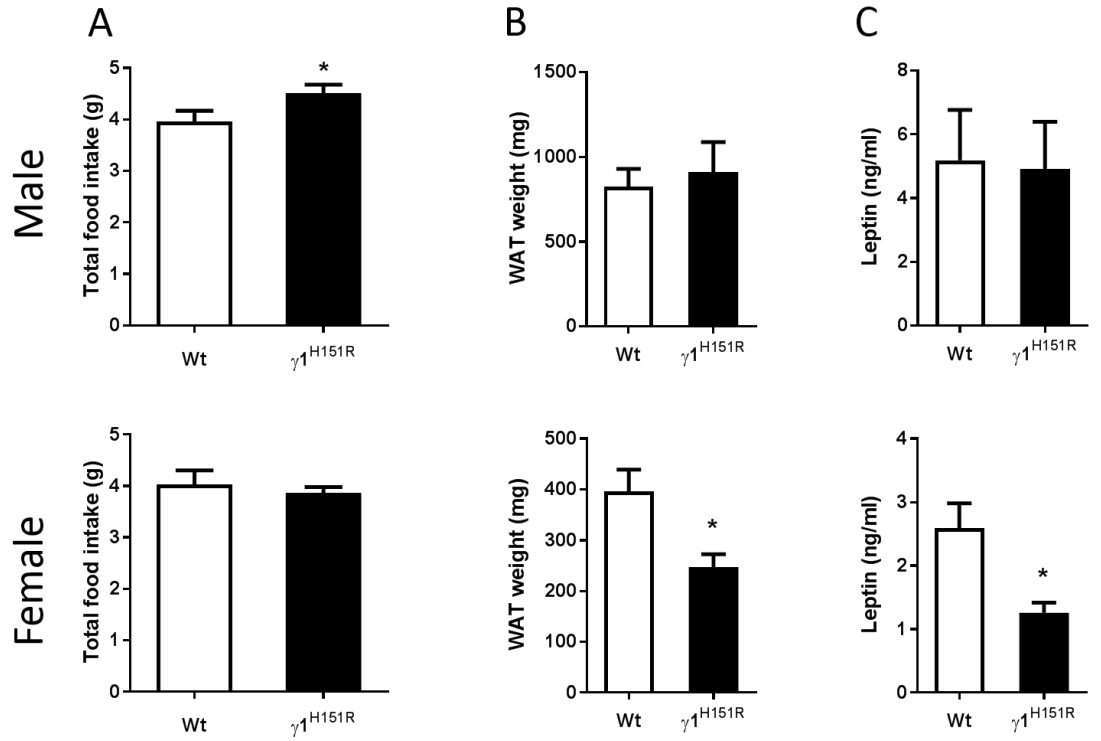

Figure 10: Sex-specific effects of skeletal muscle AMPKγ1^{H151R} expression. Food intake was measured for 2 consecutive days in male and female Wt (open bars) and AMPKγ1^{H151R} transgenic mice (black bars) (16 –22 weeks of age) housed in metabolic cages (n=3-8) (A). Gonadal fat pad weight of 4-h-fasted male and female Wt and AMPKγ1^{H151R} transgenic mice (22 weeks of age) was measured after tissue dissection (n=6-9) (B). Serum leptin was measured in the same mice (C). Results are shown means ± SE. *P ≤ 0.05. **Refers to Fig. 5/7 of paper II.**

5.2.3 Skeletal muscle-specific activation of AMPKγ3 does not impact whole-body lipid oxidation

To allow for the *in vivo* assessment of whole-body fatty acid oxidation in conscious mice, we adapted and modified a technique that has previously only been used in rat studies (Oakes et al., 1999). The method relies on the intravenous administration of ^{3}H-palmitic acid combined with a non-β-oxidizable palmitate analogue, ^{14}C-2-bromopalmitate. The β-oxidation product, $^{3}H_2O$, accumulates in blood and peripheral tissues following the infusion of the tracers. Thus, it functions as a proxy for the oxidation rate, while the presence of ^{14}C-2-bromopalmitate in the tissue represents lipid uptake. We validated the reliable quantification of whole-body fatty acid oxidation by pre-treating wildtype mice with etomoxir. The resulting blockage of CPT1-mediated transport of lipids into the mitochondria led to a robust decrease of whole-body lipid oxidation without altering the overall clearance of ^{14}C-bromopalmitate from the blood, despite decreased uptake by the oxidative tissues heart and soleus (Fig. 11). In addition to the inhibitory effect of etomoxir on lipid oxidation, additional studies to assess pharmacological compounds that induce whole-body lipid oxidation in lean mice would add a valuable positive control for the functionality of the assay.

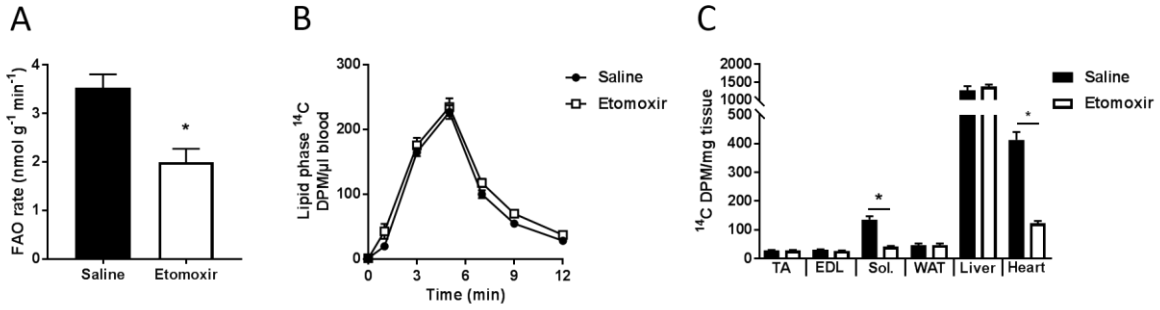

Figure 11: Validation of the technical approach to measuring fatty acid oxidation rate *in vivo*. Rate of whole-body fatty acid oxidation of saline (black bars) and etomoxir treated (open bars) mice (A). Clearance of ^{14}C-2-bromopalmitate from the blood of saline (black circles) and etomoxir-treated (open squares) mice (B). Tissue-specific uptake of non-β-oxidizable ^{14}C-2-bromopalmitate of saline- or etomoxir-treated mice (C). Results are shown as mean ± SEM, *p < 0.05. n=7 mice. **Refers to Fig. 2 of paper III.**

The recently described compound yhhu981 was shown to induce fatty acid oxidation in an AMPK-dependent manner in cell culture and to reduce RER in acutely treated *ob/ob* mice (Zeng et al., 2015). Although the impact of acute yhhu981-treatment on blood glucose was not reported, this compound could potentially provide further validation of the method presented in this study. Nevertheless, the time point for yhhu981 administration would likely have to be earlier than for etomoxir due to different pharmacodynamics. However, the chronic induction of AMPK activity in skeletal muscle of AMPKγ3^{R225Q} mice did not alter the rate of whole-body fatty acid oxidation. Although the oleate oxidation was increased in isolated EDL of high-fat fed AMPKγ3^{R225Q} mice (Barnes et al., 2004), the contribution of other tissues to whole-body fatty acid oxidation may overwrite the mild effects of AMPKγ3 activation in skeletal muscle. In particular, investigating the contribution of lipid oxidation in BAT would be of interest as this adipose tissue depot quickly responds to metabolic alterations. Chronic pharmacological β3-adrenergic stimulation of rats fed a high-fat diet increased fatty acid

utilization of BAT to a level that exceeded that of heart and liver (Warner et al., 2016). This finding illustrates the potential impact of BAT activity on whole-body lipid utilization. Future studies to investigate the impact of skeletal muscle AMPK activity on whole-body fatty acid oxidation driven by BAT could further elucidate the sex-specific effects observed in female AMPKγ1^{H151R} mice. Regardless of genotype, HFD consistently elevated whole-body fatty acid oxidation *in vivo* (Table 2). This is consistent with findings from previous studies in high-fat fed rodents focusing on fatty acid metabolism in liver and skeletal muscle (Turner et al., 2007, Ciapaite et al., 2011). However, the effect of long standing obesity and T2D on the oxidative capacity of human skeletal muscle and the potential value of therapeutically targeting it is still a matter of debate.

	FAO rate (nmol g^{-1} min^{-1})	
	WT	**AMPKγ3^{R225Q}**
Chow	4.00 ± 0.55	3.25 ± 0.30
HFD $^{\#}$	6.71 ± 0.72	5.58 ± 0.82

Table 2: $^{\#}p < 0.05$ Effect of chow or high-fat diet (HFD) on the rate of fatty acid oxidation as determined by 2-way ANOVA. Results are shown as mean ± SEM. n=5-10. **Refers to Table 2 in paper III**.

5.3 OBESITY PROMOTES ADAPTIVE CHANGES OF THE PROTEOME OF SKELETAL MUSCLE

Using a newly adapted proteomics workflow on skeletal muscle from leptin-deficient, obese and insulin resistant *ob/ob* mice we identified 118 differentially regulated proteins associated with obesity and insulin resistance (paper IV). By matching the proteomics results from quadriceps skeletal muscle to peptides and proteins detected in C2C12 myotubes, over 6,000 proteins could be identified in skeletal muscle including proteins involved in glucose and lipid metabolism, fiber type associated proteins and several secreted factors.

5.3.1 Identification of proteins involved in inter-organ crosstalk with deep proteomics

Exercise increases the secretion of multiple myokines that positively impact metabolic health (Febbraio and Pedersen, 2005). Nevertheless, the effect of obesity on the reduction of these myokines and the possible increased expression and secretion of others is incompletely understood (Oh et al., 2016). Further characterization of the inter-organ communication, especially between skeletal muscle and adipose tissue, and their dysregulation in the state of obesity and T2D could help identify new targets for the pharmacological treatment of metabolic diseases (Stanford and Goodyear, 2017). An analysis of the proteome of human adipose tissue has led to the identification of over 2,000 proteins showing differential abundance of proteins that are associated with age, sex and T2D (Gomez-Serrano et al., 2016). Such studies

underscore the value of deep proteomics techniques that enable the detection of very lowly abundant or unknown proteins in complex tissues such as skeletal muscle or adipose tissue. The here presented technical approach combining multiple enzyme digest filter-aided protein separation and MS-based peptide identification within two fractions over a relatively short reading time, in addition to the computational integration of the findings with results from cell culture models, constitutes another step towards the goal of mapping the entire proteome of cells, tissues and organisms.

5.3.2 Elevated peroxisomal and mitochondrial proteins in skeletal muscle of leptin-deficient mice

We observed an overall increase of mitochondrial proteins associated with lipid oxidation and an increased abundance of proteins characteristic of oxidative slow muscle fibers in skeletal muscle of *ob/ob* mice (Fig. 12). Additionally, elevated levels of proteins associated either with peroxisomes or with peroxisomes and mitochondria were detected, suggesting increased lipid fluxes and a concomitant elevation of cellular stress. Proteomics studies of skeletal muscle from obese men and women, on the other hand, showed reduced abundance of mitochondrial proteins and increased abundance of glycolytic proteins in obesity (Hittel et al., 2005, Hwang et al., 2010). These findings raise the question of whether human and rodent muscle responds differently to metabolic challenges such as increased availability of free fatty acids (FFA) or if different stages of disease progression can explain the differences. The general comparability of mitochondrial function between human and mouse muscle has been confirmed for quadriceps muscle of young, healthy mice and humans (Jacobs et al., 2013). Nonetheless, a comparison of our findings generated using a genetic model of obesity due to complete leptin deficiency to a model of diet induced obesity, which would arguably better reflect human obesity and the associated metabolic perturbations, would be of interest.

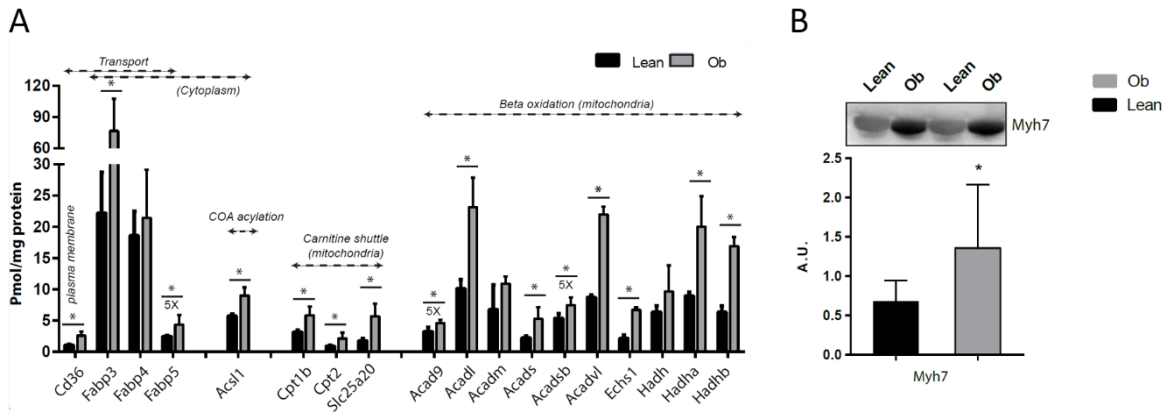

Figure 12: Enrichment of proteins associated with mitochondria and slow-twitch fibers in skeletal muscle of leptin-deficient *ob/ob* (Ob) mice. (A) Abundance of proteins associated with lipid transport, CoA acylation, carnitine shuttling and mitochondrial ß-oxidation in the proteome of quadriceps muscle of lean (black bars) and Ob mice (grey bars). Results are median ± SD, (n=4). (B) Western blot analysis of Myh7.Results are mean ± SD, (n=8). *p ≤ 0.05. **Refers to Fig. 3/5 of paper IV**.

5.3.3 Regulation of whole-body metabolic homeostasis by leptin and AMPK

We detected elevated levels of phosphorylated AMPKα^{Thr172} and ACCα/β^{Ser79} in skeletal muscle of leptin-deficient mice, which is consistent with the notion of increased lipid oxidation. Interestingly, the administration of leptin also promotes skeletal muscle fatty acid oxidation via AMPK activation and ACC inhibition (Minokoshi et al., 2002, Wolsk et al., 2011, O'Neill et al., 2014). In obese men, however, the expression of leptin receptors in skeletal muscle was downregulated, possibly indicating that direct effects of leptin on skeletal muscle are hampered in human obesity (Fuentes et al., 2010). In mice, the increased availability of free fatty acids in hyperphagic obesity may possibly stimulate lipid oxidation even in a state of complete leptin deficiency. In addition to direct peripheral effects of leptin, central leptin administration was found to stimulate AMPK activity, PGC-1α expression and glucose uptake in skeletal muscle via the hypothalamic-sympathetic nervous system axis in a PI 3-kinase-dependent manner (Roman et al., 2010). However, the effect of leptin on skeletal muscle glucose uptake appears to be AMPK independent, as it is conserved in mice expressing a dominant negative form of AMPK in skeletal muscle (AMPKα1^{D157A}) but appears to require intact β2-adrenergic signaling instead (Shiuchi et al., 2017). Indeed, increased AMPK activity in skeletal muscle of AMPKγ3^{R225Q} mice was not sufficient to ameliorate insulin-resistance and obesity caused by leptin deficiency (Zachariah Tom et al., 2014). Further studies could elucidate the extent to which the reduced plasma leptin level detected in female AMPKγ1^{H151R} mice is directly involved in the regulation of glucose and lipid metabolism in liver or adipose tissue. Leptin pre-treatment of brown adipocytes diminishes insulin-induced glucose uptake indicating that reduced exposure to leptin could increase BAT insulin action and have positive implications for the regulation of energy homeostasis (Kraus et al., 2002). Together, our findings further support the notion of a mechanistic connection between leptin action and AMPK activity in skeletal muscle in relation to whole-body energy metabolism.

6 CONCLUSION AND FUTURE PERSPECTIVE

The rapidly increasing burden of obesity and associated metabolic diseases such as type 2 diabetes on modern society underscores the need for a better understanding of the molecular processes impacting the regulation of glucose and lipid metabolism in cells, tissues and the whole body. Skeletal muscle, with its normally wide range metabolic flexibility, is a prominent target for therapeutic approaches aiming to improve metabolic health. The aim of this thesis was to further elucidate the role of skeletal muscle AMPK and DGK, two enzymes that constitute important signaling hubs in glucose and lipid metabolism, in the regulation of whole-body energy homeostasis. Additionally, by utilizing a state-of-the-art technological approach, the aim was to characterize and understand the extent to which the skeletal muscle proteome is altered in the state of extreme obesity, with a particular focus on proteins involved in energy handling.

We found that the ablation of DGKε caused an elevation of DAGs in skeletal muscle of diet-induced obese mice while concomitantly enhancing whole-body glucose tolerance and increasing the relative lipid oxidation rate. This effect was possibly due to an overall increase of lipid fluxes, thereby preventing lipotoxicity. However, skeletal muscle and hepatic insulin sensitivity were unchanged pointing towards enhanced insulin sensitivity of other tissues that have yet to be identified. Together with previous data demonstrating moderate physiological changes upon the loss of DGKα, favorable metabolic effects following the loss of DGKζ and the detrimental metabolic impact of the loss of DGKδ, our results indicate that different DGK isoforms play distinct roles in skeletal muscle and whole-body lipid and glucose metabolism. Further studies should identify the therapeutic value of isoform-specific modulations of DGK activity, for example by identifying or designing DGKδ activators and DGKε inhibitors, potentially even in a tissue-specific manner.

Overexpressing AMPKγ1^{H151R} in skeletal muscle bypassed the necessary activation of the AMPK complex through low cellular energy levels and led to improvements in insulin sensitivity and increased carbohydrate metabolism. Overall our results suggest that AMPK activation provides a potential protection against the development of insulin resistance. Future studies should evaluate whether this effect is preserved in obesity. Male mice expressing this activated form of AMPK were more active and showed an increased energy expenditure. Female mice showed a reduction of perigonadal WAT mass and leptin levels, as well as altered adipose tissue gene expression, possibly pointing towards increased BAT activity. Identifying the mechanism involved in the possible regulation of adipose tissue activity by skeletal muscle AMPK activation would be of great interest to further characterize the tissue crosstalk and its impact on whole-body energy metabolism. This is particularly important with regard to acute exercise and exercise training where the activation of AMPK is associated with positive effects on the whole body. Remaining of interest is the development of isoform-specific AMPK activators for the treatment of metabolic disease. This may be achieved by modifying recently developed decently specific AMPK activating compounds. Our data also underline the importance of investigating the effects of metabolic interventions in males and females in pre-

clinical and clinical studies given the sex differences observed in male and female mice expressing the AMPK$\gamma1^{H151R}$ transgene.

By using a newly established *in vivo* approach for measuring whole-body fatty acid oxidation in mice, employing ^{3}H-palmitate, we were able to determine that the skeletal-muscle specific expression of AMPK$\gamma3^{R225Q}$ does not alter the whole-body rate of lipid oxidation, despite enhanced palmitate oxidation in isolated glycolytic skeletal muscle. Thus, the effects of this mutation in skeletal muscle may be masked at the whole-body level, given the contribution of other tissue to the regulation of whole body metabolism. Thus, a more in-depth analysis of the contribution of a range of individual tissues to whole body lipid metabolism as assessed with this assay is warranted to provide a valuable resource for studies of metabolically active compounds and their systemic effects. The treatment of mice with the CPT1-inhibitor etomoxir, preventing lipid transport into mitochondria, consistently lowered the lipid oxidation rate providing a proof-of-concept. Interestingly, high-fat fed mice, independently of genotype, showed elevated oxidation rates compared to lean mice, arguing against impaired oxidative capacity in early obesity. Whether these changes in lipid oxidation are preserved at later stages in the course of obesity and the development of insulin resistance, or in states of excessive hepatic lipid accumulation, should be investigated in future studies.

The characterization of changes in the skeletal muscle proteome in leptin-deficient obesity provides a valuable research resource for future studies on cause and treatment of skeletal muscle insulin resistance. Of note, the mitochondrial and peroxisomal proteins involved in glucose and lipid utilization, as well as the proteins involved in oxidative stress, that were differentially abundant in skeletal muscle of lean and obese mice should be in the focus of these future investigations. Further studies are also warranted to assess whether the observed effect of leptin deficiency and obesity on the skeletal muscle proteome of male mice is comparable to that of female mice, especially with regard to the fiber type abundance and the activity of AMPK. Overall, the preservation or loss of the oxidative capacity of skeletal muscle mitochondria appears to be determining in the development and progression of insulin resistance and type 2 diabetes. Additional insight into differentially expressed known or novel secreted factors would also be valuable to understand the underlying cause of skeletal muscle insulin resistance. This would also be of interest with regard to the identification of exercise-mediated effects on the secretion of myokines in order to preserve insulin sensitivity. Here further optimization of proteomics applications is crucial to eventually allow for the measurement and identification of the complete proteome of a tissue and ideally in blood. Expecting that in the future, the time and cost of these analyses will be further reduced, these approaches may offer an invaluable tool in combination with other “omics” approaches for diagnostics of disease markers, the establishment of hallmarks of disease progression and personalized medicine.

The regulatory network orchestrating metabolic processes in the cell consists of several hundred enzymes, including a plethora of kinases. By focusing on kinases involved in the regulation of skeletal muscle metabolism with effects on the whole body, the work presented here (Fig. 13) identifies possible targets for the prevention and treatment of metabolic diseases.

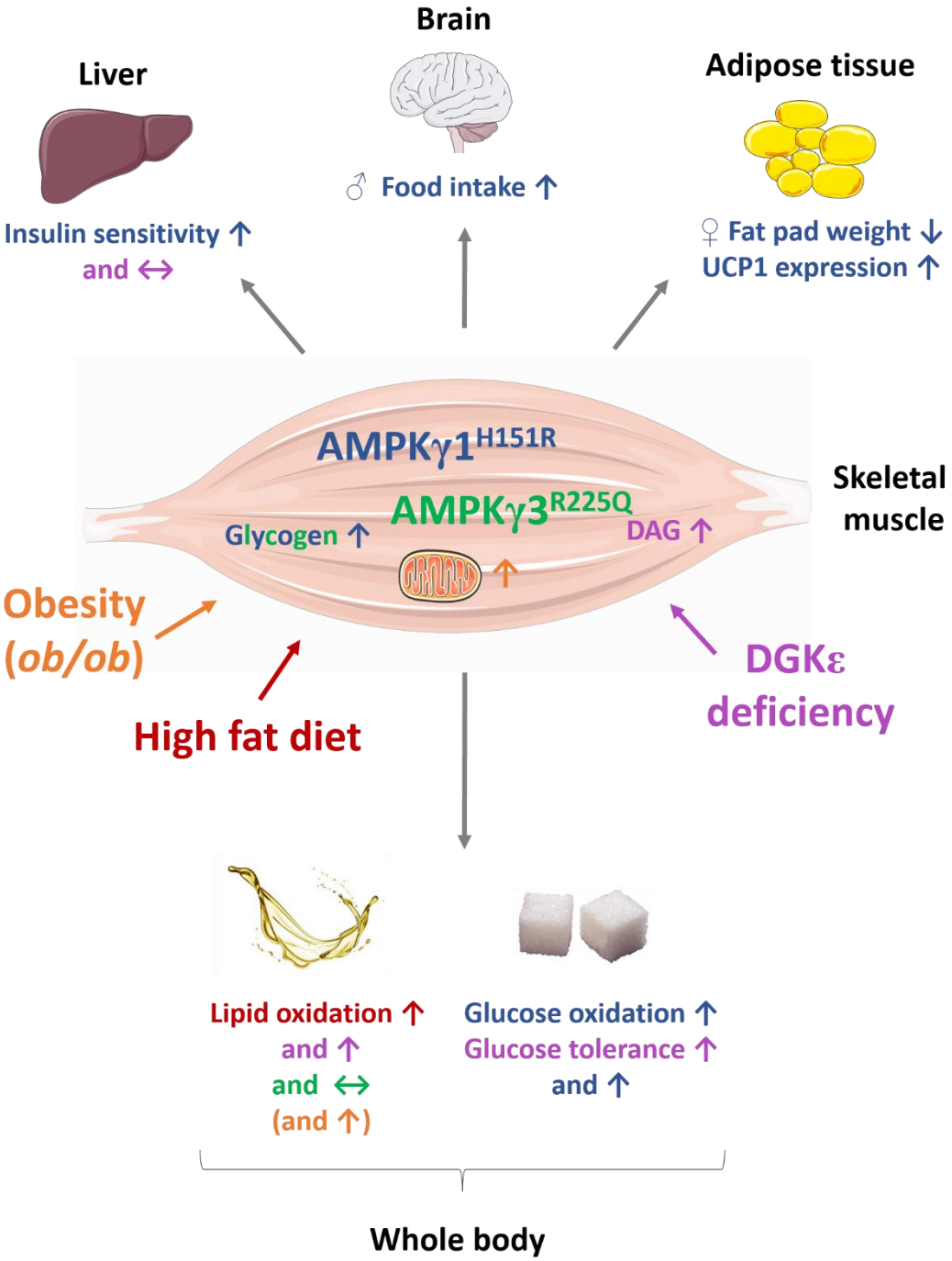

Figure 13: Schematic overview over the studies and main findings presented in this thesis.

7 ZUSAMMENFASSUNG

Übergewicht und damit assozierte Krankheiten wie Typ-2-Diabetes nehmen weltweit stetig zu. Das Gleichgewicht zwischen Nahrungsaufnahme und Energieverbrauch im Körper unterliegt einer strengen Kontrolle und wird durch verschiedene Gewebe reguliert. Quergestreifte Muskulatur ist einer der Hauptakteure in diesem regulierenden System, z.B. weil sie je nach Angebot sowohl Zucker als auch Fett problemlos verstoffwechseln kann. Dies beeinflusst den Stoffwechsel des gesamten Körpers. Eine Vielzahl Hormone und Enzyme sind in diese Prozesse im Muskel eingebunden und die hier vorliegende Arbeit befasst sich mit einigen von ihnen.

Das Enzym Diacylglycerinkinase (DGK) reguliert die Konzentration verschiedener Fette in der Zelle. Mehrere ähnliche Enzymvarianten gehören zu der DGK-Familie und wir haben untersucht, welche Rolle das Familienmitglied DGKε in der Regulierung des Stoffwechsels spielt. Das Ausschalten des DGKε-Gens in Mäusen führte zu einer Ansammlung bestimmter Fette in den Muskelzellen. Obwohl dies in der Regel mit Insulinresistenz assoziiert ist, z.b. bei starkem Übergewicht, waren die Mäuse in der Lage größere Mengen Zucker aus dem Blut in die Körpergewebe aufzunehmen. Interessanterweise bauten die Mäuse allerdings verhältnismäßig mehr Fett als Zucker ab im Vergleich zu Mäusen mit intaktem DGKε. Ingesamt zeigen diese Ergebnisse, dass DGKε sowohl Teil der Regulierung des Fettstoffwechsels im Muskel als auch des Gesamtstoffwechsels des Körpers ist.

Die Aktivierung des aus drei Untereinheiten bestehenden Enzyms AMP-aktivierte Proteinkinase (AMPK) begünstigt die Herstellung des Energieträgers ATP in der Zelle in Phasen des erhöhten Energiebedarfs wie während des Sports. Die genetische Veränderung der AMPKγ1-Untereinheit im Muskel führte zur dauerhaften Aktivierung des Enzymkomplexes mit weitreichenden Folgen für den gesamten Körper. Die Empfindlichkeit sämtlicher Gewebe gegenüber Insulin war gesteigert und die Mäuse bauten insgesamt mehr Zucker ab. Speziell in Weibchen führte diese muskelspezifische Mutation darüberhinaus zu einer Verkleinerung der Bauchfettpolster. Diese Erkenntnisse unterstreichen den potentiellen therapeutischen Nutzen von Wirkstoffen, die AMPK direkt im Muskel stimulieren. Obwohl die muskelspezifische genetische Aktivierung von AMPKγ3 ebenfalls mit Veränderungen von Stoffwechselprozessen assoziert ist, begünstigte sie in unserer Studie keine verstärkte Verstoffwechselung von Fett. Mit einer neuen Methode, basierend auf der Verstoffwechselung von radioaktivem ^{3}H-Palmitat und der anschließenden Quantifizierung des enstehenden Wassers ($^{3}H_2O$), konnten wir allerdings eine ingesamt erhöhte Fettstoffwechselrate in Mäusen auf einer fetthaltigen Diät feststellen. Ob eine fetthaltige Diät im Menschen die Fettstoffwechselrate allerdings tatsächlich erhöht oder eventuell sogar reduziert und damit eine weitere Gewichtszunahme begünstigt, ist weiterhin ungeklärt.

Die Grundstruktur des Muskels verändert sich mit Übergewicht und die Herstellung von Proteinen passt sich sowohl an das gesteigerte Energieangebot als auch das erhöhte Körpergewicht an. Mithilfe einer auf Massenspektroskopie basierenden Methode konnten wir über 6000 Proteine des "Proteoms" (die Gesamtheit aller Proteine) in den Muskeln von

schlanken und stark übergewichtigen Mäusen denen das Sättigungshormon Leptin fehlt (sog. *ob/ob* Mäuse) identifizieren. 118 dieser Proteine kamen entweder häufiger oder weniger häufig in den Muskeln der dicken im Vergleich zu denen der schlanken Mäusen vor. Insbesondere die Menge an Enzymen die Teil des Fettstoffwechsels sind und an Proteinen charakteristisch für langsame Typ I Musklefasern war größer. Desweiteren kamen zahlreiche Proteine, die typischerweise in Mitochondrien und Peroxisomen vorkommen, vermehrt vor. Diese Zellorganellen spielen eine entscheidende Rolle im Zellstoffwechsel und der Abpufferung von Zellstress. Ingesamt zeigen auch diese Ergebnisse, dass chronisches Übergewicht, zumindest in Mäusen, sehr wahrscheinlich mit einer erhöhten Fettstoffwechselrate in den Muskeln einher geht.

Zusammenfassend lässt sich sagen, dass sowohl die enzymatische Aktivität von DGKε als auch von AMPKγ1 and γ3 im Muskel Einfluss hat auf die Regulierung des Stoffwechsels des gesamten Körpers. Übergewicht verursacht weitreichende Veränderungen in diesem System und beinflusst die Herstellungsrate von bestimmten Proteinen, was möglicherweise die erhöhte Verstoffwechselung von Fett begünstigt.

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